Development of Water and Wastewater Biofiltration Technologies for the Developing World Using Locally Available Packing Media: Case Studies in Vietnam and Haiti

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
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ABSTRACT

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Abstract

Water and sanitation are two of the world's most urgent current challenges (Elimelech, 2006). With a population racing towards seven billion people, over one sixth of the human population does not have access to adequate water and sanitation. Drinking water is inaccessible for approximately 783 million people living in the developing world (WHO, 2014). This is especially critical for people at risk of exposure to deadly pathogens such as *Vibrio cholerae*, *Shigella*, and *Salmonella*, such as those living in Haiti as *Vibrio cholera*e is now ubiquitous (Enserink, 2010). On the sanitation side, more than 2.5 billion people in the world still lack access to adequate resources (WHO, 2014). Almost half of these people have access to no sanitation facilities at all and practice open defecation (WHO, 2014). Thousands of small children still die every day from preventable diseases caused by inadequate sanitation (WHO, 2014). As global climate change is expected to exacerbate these issues, there is an urgent need for the development of sustainable treatment technologies to ensure a better tomorrow for our world (Ford, 1999). Although empirical evidence is pessimistic about the benefits of integration, safe water and sanitation technologies, while often disjointed, should be considered together as pathogens transmitted via drinking water are predominantly of fecal origin (Ashbolt, 2004; Montgomery, 2007).

In this dissertation project, the use of both drinking water and wastewater treatment technologies which are cost effective and rely on locally available materials in low-income countries is explored. For the drinking water treatment side, the focus is on

the use of biosand filters in Haiti with a specific interest in understanding their ability to remove the pathogen *Vibrio cholerae*, the causative agent for cholera. The wastewater treatment technology consists of biofilters packed with cocopeat, a waste product generated during coconut husk processing, and their use for the treatment of septic tank effluent in Vietnam is investigated. Both of these projects combine lab and field work.

The specific objectives of this dissertation project are to 1) develop a method for field enumeration of *V. cholerae* (lab component); 2) perform a field survey in Haiti to determine if indicator bacteria are appropriate surrogates for estimating *V. cholerae* removal in biosand filters and identify parameters controlling *V. cholerae* removal (field component); 3) test the effect of length of operation, total organic carbon loading, and schmutzdecke composition on *V. cholerae* removal efficacy in lab biosand filters (lab component); 4) evaluate cocopeat as a packing medium for the treatment of wastewater (lab component); 5) conduct an assessment of cocopeat-packed, vertical flow constructed wetlands treating septic tank effluent in the Mekong Delta of Vietnam (field component).

In the first part of this dissertation, biosand filters in the Artibonite Valley of Haiti, the epicenter of the cholera epidemic, were tested for total coliform and *V. cholerae* removal efficiencies. In addition, schmutzdecke samples were collected in order to measure the amount of EPS in the biofilm, as well as characterize the microbial community. Total coliform and *V. cholerae* concentration were measured using novel membrane filtration technique methods. It was found that total coliform concentration does not indicate *V. cholerae* concentration in water, and total coliform removal

efficiency does not indicate *V. cholerae* removal efficiency within biosand filters. Additionally, parameters controlling biosand filter performance include: schmutzdecke composition, time in operation, and idle time.

In the second part of this dissertation, *V. cholerae* challenge tests were performed on laboratory-operated biosand filters receiving high, medium or low TOC influents in order to determine the effect of number of charges, total organic carbon loading, and schmutzdecke composition on *V. cholerae* removal efficacy, as well as to isolate the effect of biological removal mechanisms and physical/chemical removal mechanisms on *V. cholerae* removal efficiency and determine the correlation to TOC concentration in water. To this end, three biosand filters were operated in the lab. Each received lake water or diluted lake water with high, medium or low concentrations of TOC. After being charged once per day for 6 days, the filters were charged with four consecutive charges of pure cultures of *V. cholerae* suspended in PBS buffer, at concentrations of 10^2, 10^3, 10^5, and 10^7 cfu/mL. This challenge was repeated each time the filters received an additional 6 charges, up to 66 total charges. This was done to determine how number of charges, TOC loading, and schmutzdecke composition affects removal efficiency. Schmutzdecke was analyzed for amount of EPS and microbial community. It was found that parameters controlling biosand filter performance include: TOC loading, schmutzdecke composition, time in operation, and physical/chemical attachment. Additionally, it was shown that physical/chemical attachment is critical during startup, especially at low TOC concentrations. At steady state, physical/chemical attachment is more important than

schmutzdecke effects in filters receiving low TOC, and schmutzdecke effect is more important than physical/chemical attachment in filters receiving high TOC.

For the third section of this dissertation, columns packed with cocopeat, celite, or sphagnum peat were charged with simulated wastewater and removal efficiencies of nitrogen, phosphorus, and biological oxygen demand were measured. Additionally, different redox zones were tested to determine if cocopeat could successfully accomplish nitrification and denitrification. It was found that cocopeat is comparable to traditional packing media and can successfully accomplish nitrification and denitrification in the treatment of synthetic wastewater.

In the final section of this dissertation, constructed wetlands were built and packed with cocopeat to determine if cocopeat is a suitable packing media in constructed wetlands treating wastewater in Vietnam. Removal efficiencies of nitrogen, phosphorus, and biological demand were measured. Microbial community samples were collected periodically in order to analyze community shifts between wetlands and over time. This work concluded that cocopeat can be used successfully as a packing media in constructed wetlands treating wastewater for the removal of nitrogen, phosphorus, and total coliform.

Overall, this dissertation work contributes to the body of knowledge on point-of-use water and wastewater technologies. The biosand filter was studied in both lab and field conditions and it was found that total coliform is not a reliable indicator for *V. cholerae*, and that there are several factors controlling biosand filter performance, including idle time, TOC, filter time in operation, physical/chemical attachment, and

schmutzdecke composition. Cocopeat was studied for its ability to promote nitrification and denitrification in lab-scale vertical flow columns treating synthetic wastewater. It was shown that cocopeat achieved similar levels of nitrification and denitrification as traditional packing media. Finally, cocopeat packed vertical flow constructed wetlands were operated in Vietnam for the treatment of septic tank effluent. This setup proved effective for the removal of nitrogen, phosphorus, and total coliform in the treatment of wastewater.

Dedication

This dissertation is dedicated to my parents, Dirk and Jeri Danley, for their love, support, and dedication to making education a priority and value in my life. It has all led to this - thank you.

"I lift my eyes up to the hills – where does my help come from? My help comes from the LORD, the Maker of heaven and earth." -- Psalm 121: 1-2

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Chapter 1. Introduction

Water and sanitation are two of the world's most urgent current challenges (Elimelech, 2006). With a population racing towards seven billion people, over one sixth of the human population does not have access to adequate water and sanitation (as defined by the World Bank). As global climate change is expected to exacerbate these issues, there is an urgent need for the development of sustainable treatment technologies to ensure a better tomorrow for our world (Ford, 1999).

Drinking water is inaccessible for approximately 783 million people living in the developing world (WHO, 2014). This is especially critical for people at risk of exposure to deadly pathogens. The average child in the developing world suffers from 3 or more diarrheal cases each year, causing over 4 billion incidents in children annually (Stauber, 2009). Diarrheal disease constitutes 4% of the global burden of disease and kills more than 1 million people every year (Stauber, 2009). Three bacteria of concern are *Salmonella spp.*, *Shigella spp.* and *Vibrio cholerae*, the bacteria responsible for the illnesses salmonella, dysentery and cholera, respectively. The infectious dose for these bacteria can be seen in Table 1.1 (Ford, 1999).

Table 1.1 Pathogens in Drinking Water (Ford, 1999)

Bacteria	Infectious Dose	Estimated Incidence (1/yr)	Survival in Drinking Water (d)
Vibrio cholerae	1.E+08	very few	30
Salmonella spp.	1.E+06	59,000	60-90
Shigella spp.	1.E+02	35,000	30

On the sanitation side, more than 2.5 billion people in the world still lack access to adequate resources (WHO, 2014). Almost half of these people have access to no sanitation facilities at all and practice open defecation (WHO, 2014). Thousands of small children still die every day from preventable diseases caused by inadequate sanitation (WHO, 2014). These diseases are caused predominantly by rotavirus, *Campylobacter jejuni*, enterotoxigenic, *Escherichia coli*, *Shigella* spp. and *V. cholerae* O1, and possibly enteropathogenic *E. coli*, *Aeromonas* spp., *V. cholerae* O139, enterotoxigenic *Bacteroides fragilis*, *Clostridium difficile* and *Cryptosporidium parvum* (Ashbolt, 2004).

Although empirical evidence is pessimistic about the benefits of integration, safe water and sanitation technologies, while often disjointed, should be considered together as pathogens transmitted via drinking water are predominantly of fecal origin (Ashbolt, 2004; Montgomery, 2007). A meta-analysis of the impact of interventions showed that point-of-use (POU) water treatment technologies and improved sanitation led to 35 and 32% reductions in diarrhea, respectively (Fewtrell et al, 2005). These two improvements

resulted in great diarrheal reductions, possibly because they directly block pathways of exposure (Montgomery et al, 2007).

As discussed above, while progress has been made in both the availability of water and wastewater treatment in the developing world, there are substantial improvements still needed. In this dissertation project, the use of both drinking water and wastewater treatment technologies was explored with a special emphasis on solutions which are cost effective and rely on locally available materials in low-income countries. For the drinking water treatment side, the use of biosand filters was investigated in Haiti with a specific interest in understanding their ability to remove the pathogen *Vibrio cholerae*, the causative agent for cholera. In addition, a wastewater treatment technology consisting of biofilters packed with cocopeat, a waste product generated during coconut husk processing, was investigated for the treatment of septic tank effluent in Vietnam. Both of these projects combined laboratory and field work components. The specific objectives of this dissertation were to:

1) Develop a method for field enumeration of *V. cholerae* (lab component)
2) Determine if indicator bacteria are appropriate surrogates for estimating *V. cholerae* removal in biosand filters and identify parameters controlling *V. cholerae* removal (field component)
3) Test the effect of length of operation, total organic carbon loading, and schmutzdecke composition on *V. cholerae* removal efficacy in lab biosand filters (lab component)

4) Evaluate cocopeat as a packing medium for the treatment of wastewater (lab component)
5) Conduct an assessment of cocopeat-packed, vertical flow constructed wetlands treating septic tank effluent in the Mekong Delta of Vietnam (field component).

Research Hypotheses and Approach

This dissertation was broken down into two overall objectives. The first was to better characterize biosand filter operation in terms of pathogen removal and parameters affecting biosand filter performance, especially during the startup phase. The second overall objective was to evaluate cocopeat as a biofiltration packing medium as compared to traditional packing media and evaluate its performance in a real-world setting.

The first objective of this dissertation was to develop a method for field enumeration of *V. cholerae*. While coliform are often used as an indicator organism for pathogenic bacteria, a correlation has never been established linking coliform and *V. cholerae* presence and it has been shown that indicator bacteria do not always accurately represent concentrations of specific pathogens of interest. Because indicator organisms such as *E. coli*, total coliform, and fecal coliform may not be appropriate indicators for pathogens of interest, there is a need for the development of field based methods to quantify pathogens. In particular, most efforts have focused on coliform with little work on other species. This is problematic as a correlation does not always exist between indicator bacteria and the pathogenic target. Thus, there is a clear need for better field methods. In the present study, a plate count test utilizing membrane filtration technique

was developed which could be applied for the measurement of viable *V. cholerae* cell concentration in the field. Method accuracy was confirmed by comparing plate count concentrations to hemocytometer (microscope) concentrations.

The second objective was to test biosand filters in Haiti and compare the removal efficiency of *V. cholerae* to indicator bacteria in field biosand filters as well as investigate the correlation between removal efficiency and time in operation, schmutzdecke bacterial composition, and influent water characteristics. There were two overall hypotheses addressing this objective. The first was that biosand filter removal efficiencies of specific pathogens would not correlate well to indicator organism removal efficiency. This hypothesis was based on the fact that bacteria have different surface properties that will affect attachment efficiencies in a biofiltration process. Total and fecal coliform are commonly used as indicator organisms to predict the presence of other pathogenic bacteria as they are generally spread via fecal contamination. However, a correlation has never been established linking coliform and *V. cholerae* presence. The second hypothesis was that time in operation, schmutzdecke composition, and influent water characteristics will impact biosand filter performance. The formation of the schmutzdecke is known to be critical for biosand filter operation as the microorganisms degrade organic contaminants and the layer also acts as a filtration step. The removal of this biolayer has previously been shown to adversely impact treatment efficacy, however little work has been performed to characterize this biolayer. Also, total organic carbon loading varies substantially between water sources, which could affect biosand filter performance. The working hypotheses were that: 1) total coliform is not a good indicator of the

concentration of specific pathogenic bacteria, and 2) biofilter ripening and hence schmutzdecke characteristics control treatment efficiency. Organic loading is critical upon startup to develop the required biofilm, however with increasing operation time, this parameter becomes less important relative to treatment efficacy as the schmutzdecke is already established. To test these hypotheses, biosand filters in the Artibonite Valley of Haiti, the epicenter of the cholera epidemic, were tested for total coliform and *V. cholerae* removal efficiencies. In addition, schmutzdecke samples were collected in order to measure the amount of EPS in the biofilm, as well as characterize the microbial community. Total coliform and *V. cholerae* concentration were measured using novel membrane filtration technique methods, described in the first objective.

Objective 3 involved performing *V. cholerae* challenge tests on laboratory-operated biosand filters receiving high, medium or low TOC influents in order to determine the effect of number of charges, total organic carbon loading, and schmutzdecke composition on *V. cholerae* removal efficacy, as well as to isolate the effect of biological removal mechanisms and physical/chemical removal mechanisms on *V. cholerae* removal efficiency and determine the correlation to TOC concentration in water. The working hypotheses were: 1) the filter receiving low TOC influent would experience greater *V. cholerae* removal efficiency due to the effect of physical/chemical attachment, and 2) schmutzdecke will play a larger role in removal efficiency in filters receiving high TOC than low TOC because TOC nourishes a growing biofilm. To test these hypotheses, three biosand filters were operated in the lab. Each received lake water or diluted lake water with high, medium or low concentrations of TOC. After being

charged once per day for 6 days, the filters were charged with four consecutive charges of pure cultures of *V. cholerae* suspended in PBS buffer, at concentrations of 10^2, 10^3, 10^5, and 10^7 cfu/mL. This challenge was repeated each time the filters received an additional 6 charges, up to 66 total charges. This was done to determine how the number of charges, TOC loading, and schmutzdecke composition affects removal efficiency. Schmutzdecke was analyzed for amount of EPS and microbial community distribution.

Objective 4 was carried out in upright columns packed with cocopeat, celite (an inert clay pellet), or sphagnum peat (a traditional packing media). The working hypothesis was that cocopeat treatment effectiveness is similar to other packing media effectiveness in biofiltration columns treating wastewater. While cocopeat is a sustainable and locally available packing medium for biofiltration, it is necessary to evaluate how it compares to other packing media. To test this hypothesis, columns were charged with simulated wastewater and removal efficiencies of nitrogen, phosphorus, and biological oxygen demand were measured. Additionally, different redox zones were tested to determine if cocopeat could successfully accomplish nitrification and denitrification.

Objective 5 was carried out in Vietnam, where constructed wetlands were built and packed with cocopeat to determine if cocopeat was a suitable packing media in constructed wetlands treating wastewater. Removal efficiencies of nitrogen, phosphorus, and biological demand were measured. Microbial community samples were collected periodically in order to analyze community shifts between wetlands and over time. The working hypothesis for this objective was: cocopeat is a suitable material for biofiltration

that can accomplish effective removal efficiencies of nitrogen, phosphorus, and total coliform in a scaled-up field bioreactor for the long term treatment of septic tank effluent.

Chapter 2. Literature Review

Access to water and wastewater treatment are two of the world's most pressing issues. Without safe drinking water, people are vulnerable to exposure to pathogens. Without adequate sanitation, drinking water sources are at a high risk for contamination by deadly pathogens. Biosand filters are one point of use technology that has been used in the developing world for treating drinking water at the household level. Thousands are currently in use in Haiti, where inadequate water and sanitation resources has contributed to the spread of cholera. There are several technologies available for sanitary wastewater treatment, one of which includes biofiltration. Naturally occurring media, such as peat, can be used to remove nutrients and pathogens from wastewater by filtration. In Vietnam, cocopeat is currently being investigated to assess the removal efficacy in wastewater treatment using constructed wetlands. In the following paragraphs, an overview is given of technologies currently available for water and wastewater treatment in developing countries, as well as an overview on nitrification, denitrification, and constructed wetlands.

2.1 Drinking water in developing countries

Decreased infant mortality, increased life expectancy and health are all related to increased development and prosperity. Drinking water quality affects all three of these categories. Improved water quality could lead to increased longevity and better health because the tenor of water borne diseases will decrease. The World Health Organization

(WHO) has set basic standards for drinking water quality in developing nations. These standards are not enforced; they are guidelines. The third volume of "Guidelines for Drinking-water Quality" created by WHO explains requirements to ensure drinking water safety and minimal procedures to treat water (Guidelines, 2006).

According to the WHO, the most common and deadly pollutants in water in developing countries are those of biological origin. One study estimates that approximately 10 waterborne microbes are responsible for over 28 billion disease episodes every year in developing countries. These microbes include rotavirus, *Campylobacter jejuni*, enterotoxigenic *E. coli*, *Shigella* spp. and *V. cholerae* O1, and possibly enteropathogenic *E. coli*, *Aeromonas* spp., *V. cholerae* O139, enterotoxigenic *B. fragilis*, *C. difficile* and *C. parvum* (Ashbolt, 2004). Diarrhea, ascariasis, dracunculisis, hookworm, schistosomiasis and tracoma are among the most common diseases. The latter five diseases listed are caused by helminthes. Diarrhea can be caused by many pathogens, mostly of bacterial or helminthic origin. There is no minimal level of tolerable pathogen contaminant suggested by the WHO (Gadgil, 2008).

2.2 Common point-of-use drinking water technologies

There are a wide range of different water treatment options which are currently being employed in less developed countries. These are reviewed below.

Coagulation. In the United States, when surface water is used for drinking, bathing and other domestic purposes, it typically goes through a coagulation and

flocculation process. However, in developing countries the typical materials used for coagulation, namely ferric salts or alum, are not readily available because of the high cost of importing these chemicals. Several studies have shown that alternative materials such as the seeds of the *Moringa Oleifera* tree, a native of India, act as coagulants and antimicrobial agents when added to water (Doerr, 2005). They can also be used to reduce turbidity and hardness (Ndabigengesere, 1998).

A comparison between alum and *M. Olieifera* seeds revealed that *M. Olieifera* seeds are a viable substitute for alum in treating water, and even presents additional advantages over alum. The study reports that the optimal dosage of the shelled *M. Oleifera* seeds is equal to that of the alum. That is, when 1 mL of alum was added to 105 JTU (Jackson Turbidity Unit) water, the turbidity was decreased by 90% to 10 JTU. When 1 mL of *M. Oleifera* seeds was added to water, differences were not statistically significant. This suggests that the *M. Oleifera* seeds are a comparable coagulant to alum. It should be noted that the EPA standards for drinking water state that the water should have a turbidity of less than 1 JTU, so sedimentation alone may not be enough to remove flocs from water. A follow-up filtration step may be required (Ndabigengesere, 1998).

Nonetheless, there are drawbacks to using *M. Oleifera* seeds as coagulants. If the flocculation process is too lengthy, there is a chance of secondary bacterial growth. Also, the flocs that float to the bottom must not be consumed. They are full of contaminants, so it is essential to remove the flocs before drinking the water. It is also vital for users to know that the *Moringa* treatment does not remove 100% of the pathogens in the water

(Doerr, 2005). If additional disinfection means are available, such as chlorination or solar sterilization, they should also be utilized for optimum removal of pathogens.

M. Oleifera seed coagulation can be implemented locally with readily available resources. This process could be used at a household level scale. Two *M. Oleifera* trees can provide a sufficient amount of seeds to supply a family of five for their water treatment needs (Doerr, 2005). Since *M. Oleifera* trees can grow in most tropical places, such as Haiti and Vietnam, this process could potentially be a long-term solution to the water treatment problem. However, users need training and education prior to the usage of this technology in order to ensure proper mixing speeds and times are utilized. Additionally, in order to limit exposure to pathogens, consistent treatment must be provided, and high adherence maintained.

Other coagulation technologies are also widely available, such as PUR packets. These packets utilize coagulation to remove suspended solids and calcium hypochlorite to disinfect.

Filtration. Filtration is a commonly used water treatment technology which is used to remove smaller flocs that are unable to be removed by sedimentation. One commonly used filtration device is a slow sand filter. The sand traps particles and pollutants, separating them from the water. In developing countries, slow sand filters are not always practical because they are very large and they are typically designed to treat water on a large-scale in the context of a water treatment plants. However, sand filters can also be used to treat water on a small-scale.

The biosand filter, a variation on the slow sand filter design, is a small-scale, household system. In addition to its ability to filter out flocs, this system also intercepts helminthes, protozoa and larger particles. A biofilm layer develops at the top of the biosand filter which facilitates the removal of bacteria and viruses. Many of the organisms that gather in the biofilm are eliminated by predation and organic materials in the water become part of the food chain that is active in the biofilm (Sobsey, 2008). A more detailed description of the biosand filter is provided in a subsequent section.

Another filtration alternative is to use ceramic as a filter. One type of filter that uses ceramic is a terracotta pot. The ceramic pot is made from terracotta clay and sawdust. After being formed into a large flower pot shape, it is kilned at 900ºC. The high temperature burns off the sawdust which leaves pores for water to pass through. After this, the pot is dipped into colloidal silver, which acts as a disinfectant. The pot is then placed inside the lid of a plastic container. To use this filter, water is poured in the top. The water is driven by gravity and it passes through the tiny holes in the clay, and then falls down into the plastic container, which acts as a safe storage place for the water. Finally, a spigot is attached to the bottom of the plastic container forming a fully enclosed system, but allowing for easy access to the treated water. Ceramic filters coated in colloidal silver accomplish two processes in one: filtration and disinfection (Brown, 2007).

There are also more conventional filtration technologies generally available in developing countries, such as Aquaguard, although they tend to be more expensive.

And again, the challenges with these technologies include behavioral ones. Issues such as breakage, cost of awaiting water, concerns over taste and space taken up by filters present challenges.

Disinfection. Disinfection technology is critical for the removal of some harmful pathogens from water. Chlorine and its derivatives are most often used in traditional, large-scale water treatment because of their effectiveness, low cost and residual effect. Chlorine can also be used in developing countries. If chlorine is not available, bleach (sodium hypochlorite) is an appropriate alternative. It is common practice to use one teaspoon of bleach for every 25 liters of untreated water, with effectiveness varying with water quality. A study was done in Kenya where bleach was added to household water storage containers. The bleach lowered the turbidity of the water and decreased the occurrences of diarrhea by 25% among participants. However, user uptake is often low because adding bleach to water alters taste (Tumwine, 2005). Also, if the water is exposed to direct sunlight within two hours of adding bleach, the UV could break down the residual bleach and the water could become contaminated again. Disinfection using bleach is a chemical process and it can be used on a small-scale for household or on a large-scale for a city or village. There are, of course, behavior challenges here, especially in regards to discontinued use of chlorine due to taste adversions.

Solar disinfection (SODIS) is another alternative which can be used by those with no access to chlorine or bleach. The process includes placing water in an airtight, transparent container (usually a plastic bottle) and shaking it every hour. The bottle

should be placed in an area of direct sunlight for at least six hours. Placing the container on top of a tin roof is ideal because the sunlight hits the container and is reflected off the roof, hitting the container again. The sunlight treats the water using UV-A radiation (wavelength 320-400 nm) and increased water temperature. The higher the temperature rises, the faster and more effective the process. In fact, if the temperature rises above 122ºF, the disinfection process is three times faster than that of the process at 70ºF (Ubomba, 2008).

Boiling water or pasteurization is another disinfection alternative. The high temperature debilitates and kills bacteria and harmful pathogens. Unfortunately, in order to boil water, much energy is needed. On average, 1 kg of wood to is required to boil 1 liter of water. In addition, wood and biomass fuels are not always available, and excessive amounts of fire can deteriorate the air quality in the region. Moreover, burning wood can lead to deforestation (Clasen, 2008). There are also behavioral challenges with boiling.

It should be noted that all of these technologies are only as effective as subsequent behavior entails. For example, recontamination commonly occurs in storage containers or due to unhygienic removal of safe water from storage.

2.3 Biosand filter

In this dissertation project, biosand filters were selected as the primary treatment technology because they are inexpensive, have a long-life, and are low maintenance. As of 2008, biosand filters had been installed in more than 80,000 homes around the world

(Stauber, 2009). It has been shown that biosand filters can effectively remove almost 100% of particulate matter, 100% of parasites, helminthes and larger organisms, 90-99% of viruses and bacteria, 99% of iron and manganese, substantial amounts of dissolved organic solids, 50% of inorganic toxins and considerable amounts of undesired fluoride. It costs about $40 US to manufacture and install the biosand filter. Its average life is about 20 years (Clean Water for Haiti, 2010). Biosand filters are simple to operate and require no pumps because they are gravity-driven (Fewster, 2010).

Setup. Figure 2.1 shows a schematic of a biosand filter. The water flows gravimetrically, first through a fine sand layer and then travels through coarse sand and gravel. Most of the treatment occurs in the fine sand layer where chemical and microbial contaminants can be removed by sorption or biodegradation. As water passes through the filter, microorganisms such as parasites, bacteria and viruses are strained and adsorbed to the sand particles. Most of this attachment occurs near the top of the sand layer, so the microbial population grows very dense. This population forms a biofilm known as the 'schmutzdecke'. The schmutzdecke removes microorganisms and increases the removal of particulate matter. Free floating bacteria and viruses attach to the top most part of the sand media, helping form the schmutzdecke. Bacteria and viruses that escape the schmutzdecke can be deactivated near the bottom of the sand column due to the lack of oxygen (Manz, 2007).

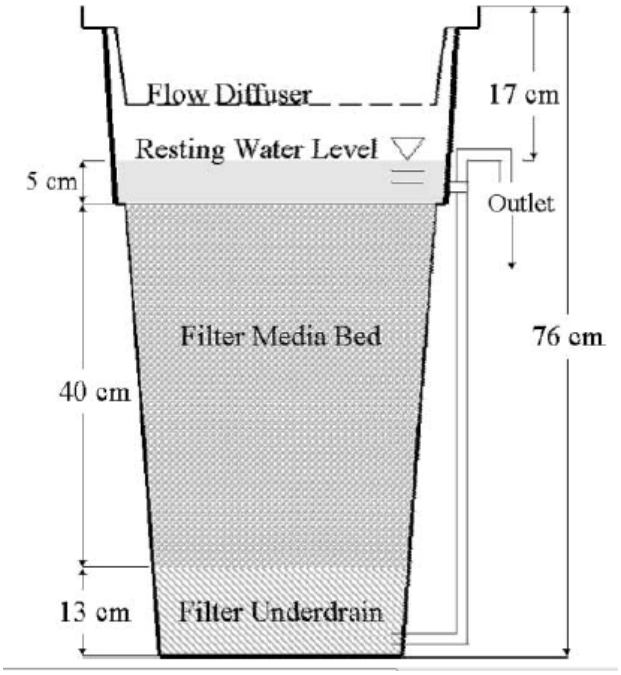


Figure 2.1 Cross-sectional view of a typical biosand filter (Stauber, 2009)

Schmutzdeck and EPS. The structure of the schmutzdecke following the initial construction of a biosand filter, as well as its properties following a filter's long term operation, are known to be critical to the quality of the drinking water effluent. However, little field research has been performed to characterize the schmutzdecke layer and determine what properties control the quality of water effluent. Furthermore, the effect of the length of idle time (i.e., the amount of time water sits in the filter prior to discharge) has not been characterized.

An understanding of bacterial surface properties is important in understanding the adhesion process of bacteria to filtration media. Three of the most important factors of

bacterial attachment to filter media are hydrophobicity, surface free energy, and surface charge (Gallardo-Moreno, 2003).

Furthermore, it has been shown that attachment stimulates bacteria extracellular polymeric substance (EPS) synthesis. Bacteria isolated from a Florida aquifer were suspended in liquid medium. When sand was added, EPS synthesis increased (Vandevivere, 1995). EPS are a major contributor to biofouling in water treatment. In biofilm, EPS forms a hydrated gel that is mainly composed of polymers such as polysaccharides, proteins, lipids, nucleic acids and humic substances (Karunakaran, 2011). It has been said that EPS "cement[s] the bacterial cells to the substrates and also aid[s] intercellular adhesion" (Karunakaran, 2011). EPS can be very beneficial in biosand filters as the generation of EPS will slow the flow rate of water through the reactor, increasing contact time with the schmutzdecke. Additionally, EPS acts as sorption sites for pollutants such as organic molecules (Flemming, 2001). EPS gives biofilms stability and can protect the biofilm from harmful chemicals, such as cleaning reagents, and predators, such as protozoa.

Biofilms are formed in five defined stages:

1. Initial reversible attachment of bacterium to substrate;
2. Irreversible attachment and microcolony formation;
3. Early development into biofilms;

4. Biofilm maturation;
5. Biofilm dispersion (Stoodley, 2002).

The initial attachment in steps 1 and 2 depend on both short- and long-range forces. Adhesion of bacteria to surfaces depends on such factors as: attractive or repulsive electrostatic forces, thermodynamic potential of the bacteria surface and the substratum, and van der Waals attraction causing polar properties (Karunakaran, 2011). *V. cholerae* 01 forms biofilms in a step-wise manner. They first contact a surface using flagellar motility and a type IV pilus. After contact is made, the sessile population grows and produces an exopolymer matrix of *Vibrio* polysaccharide (VPS) (Mueller, 2007).

These attachment mechanisms are important to consider as each bacterium will behave differently (i.e., pathogenic bacteria will have different attachment rates and mechanisms than indicator bacteria). In this study, some of these factors will be observed by measuring sorption reaction coefficients in a plug flow reactor.

Other removal mechanisms. Furthermore, while it is known that various transport mechanisms are used to remove particles from water inside a biosand filter including interception, straining, diffusion, sedimentation and hydrodynamic streamlines (Fewster, 2010), the biosand filter's ability to remove contaminants is greatly dependent on the time requirement of filter ripening and the daily volume charged to the filter (Elliott, 2008). Results from Elliott et al. (2008) show that as the filter ripening process progresses with time, the turbidity of the filtered water decreases. This indicates increased ripening

and schmutzdecke formation enhances particle straining, increases filtration throughout the depth by slowing filtration, and alters the surface properties of the filtration media. However, due to the higher filtration rate and shorter bed depth characteristic of the biosand filter, turbidity removal is lower in biosand filters than in slow sand filters (Stauber, 2006).

Ripening is the time period before a steady removal efficiency has been reached in which the biofilm is forming. The traditional ripening method involves passing a certain number of buckets of water. For example, Clean Water for Haiti recommends users pass six buckets of water through the filter before consuming the water. However, there is a large research gap here as the number of buckets to ripen the filter will depend on numerous variables such as total organic carbon (TOC).

Increased residence time and biofilm maturation have been shown to be linked to increased *Escherichia coli* removal. Also, as the filtration rate decreases with the filter age, there is an increased rate of microbial reduction. This is probably due to the longer residence time of the last parcels of water as compared to the first parcels of water (Elliott, 2008). Note that there is a particularly difficult behavioral response here. As the filter begins to work more slowly, removal is better, but user patience is challenged. Thus users feel an urge to clean the filter to make it work faster, often done by stirring the schmutzdecke, restarting the ripening process with lower removal efficiency.

It was shown that the biosand filter causes greater reduction in human enteric virus than for bacteriophages (MS2 and PRD-1). Although reductions of echovirus 12 were comparable to *E. coli* reductions, both bacteriophage reductions were uniformly

lower than *E. coli* which indicates that *E. coli* may not be a sufficient indicator for biosand filter reduction of certain viruses (Elliott, 2008).

Stauber et al (2006) showed that although biosand filter reductions of bacteria, viruses and turbidity are lower than that of traditional slow sand filters, the reductions can be improved by repeated charges, time in use as the filters mature, and increased retention time. In this study, microbes were not eliminated at the same efficiency, suggesting that there are multiple mechanisms of removal (Stauber, 2006).

2.4 Water issues in Haiti

*V. cholera*e is now ubiquitous in Haiti. This bacterium was found in every water source tested in the Haiti study conducted during this dissertation work. The cholera outbreak in Haiti began in late October 2010, just 9 months after the 7.0 earthquake struck the already fragile country (Walton, 2011). The earthquake killed over 250,000 people, injured an additional 300,000 and left more than 1.3 million homeless. Health experts warned that an outbreak of an epidemic disease was likely, due to the crowded camps of internally displaced people and the lack of proper sanitation. In August 2010, the Center for Disease Control commented on the suspicious absence of such an epidemic. However, the first cases of cholera were observed 55 miles from the capital near St. Marc on October 20, 2010 (Walton, 2011). Many more cases were reported at nearby hospitals in the next days. Between October 20 and November 9, over 11,000 cases were reported. Of these, 724 ended in death. Due, in part, to the lack of adequate sanitation and water treatment technologies, the epidemic spread. Community health

workers set up medical centers, instructions of personal hygiene were wide-spread, body bags were distributed to community leaders and throughout the countryside, rehydration centers were setup (Walton, 2011). To date, over 93,000 people have been sickened by the epidemic and over 2,100 have died (Chin, 2011).

Since Haiti had not been affected by cholera in over a century, there was a significant amount of investigation to determine the origin (Chin, 2011). After investigation by a team of scientists assembled by the UN, it was concluded that the epidemic began in a camp composed of Nepalese UN peacekeepers. The sanitation conditions were inadequate to prevent fecal contamination of the neighboring river which is a local water source for bathing, cooking and drinking. The septic waste from this camp was deposited into an open septic pit which was in close proximity to the local river (Frerichs, 2012). This incident supports the claim that sanitation and water quality cannot be separated – they are intimately linked, as poor sanitation practices leads to inadequate water quality.

A large number of biosand filters have been installed in Haiti, mostly due to the efforts of the not for profit organization Clean Water for Haiti. However, biosand filters have never been studied for the removal efficiency of *Vibrio cholerae*, the causative agent for cholera, which recently has become of interest to biosand filter users there. This topic will be addressed in my dissertation research.

2.5 Vibrio cholerae

The disease cholera causes massive fluid loss and 50% of untreated cases are fatal. However, if the lost fluids and ions are rapidly replaced, the fatalities decrease to

less than 1%. Nevertheless, cholera causes remarkably high morbidity rates and severe social and economic impacts (Winans, 2008). Throughout history, cholera has been the cause of a myriad of epidemic outbreaks and deaths. Indeed, cholera causes more than 120,000 deaths every year, mostly in children and mostly in developing countries in South Asia, Africa and Latin America (Winans, 2008). Cholera outbreaks occur in seasonal patterns and are associated with poverty and poor sanitation. Cholera outbreaks increase with the warm water cycles of El Niño, which have been steadily increasing in number since the 1970's (Beck, 2000).

The pathogenesis of *V. cholerae* begins with consumption of contaminated food or water. After surviving the harsh conditions of the stomach, the bacterium reaches the small intestine. Several gene encoding virulence factors aid the pathogen in reaching and colonizing the epithelial surface of the small intestine. Toxic proteins are produced immediately, changing the normal function of intestinal lining cells, and causing diarrheal disease in the infected individual. The bacteria leave the body with the high volumes of excreta and return to the environment where it may eventually be transferred to another human via food or water (Zhu, 2006). This is especially prone to happen in developing countries, particularly in densely populated areas.

V. cholerae has a poor acid tolerance, therefore, it can survive in bottled water, but not in carbonated water. It also thrives more on pH neutral food as opposed to acidic food. Moreover, it can survive refrigeration and freezing, which could affect food supplies that are shipped internationally. This allows for cholera to spread to countries outside the infected country (Reidl, 2002)

V. cholerae are biofilm associated bacteria, which are generally associated with chronic infection and are less susceptible than free swimming bacteria to host immunity system response and antimicrobial agents (Merrell, 2002). The infectious dose of *V. cholerae* is relatively high due to the acid-sensitivity of the bacteria and varies between 10^6 and 10^{11} cfu (Reidl, 2002).

Cholerae toxin (CT) and toxin co-regulated pilus (TCP) are two important virulent mechanisms *V. cholerae* uses in the intestine. Cholera toxin is the device that causes diarrhea in infected people by expulsing water from the lumen (Winans, 2008). Fluids are expulsed because of osmotic equilibrium. The body tries to replace the lost fluids from blood and tissue, causing a massive fluid and electrolyte loss. The greatest fluid loss occurs in the jejunum of the small intestine, where the pH is slightly alkaline. In fact, an adult can lose up to 20 L of fluid per day when infected with cholera (Reidl, 2002). TCP is a type IV pilus, which is believed to mediate the adherence of the bacterium to the intestinal mucosal cells and intestinal epithelial cells (Winans, 2008). TCP is said to be the most important colonization factor. It is thought that TCP facilitates microcolony formation and that CT does not contribute to intestinal colonization at all (Reidl, 2002). These *V. cholerae* specific mechanisms are important because the genes that encode for these can be targeted when trying to quantify *V. cholerae* with genetic methods.

2.6 Sanitation in developing countries

While access to clean drinking water is crucial, thousands of people die every day from preventable diseases caused by inadequate sanitation. Thus, radical improvements

which can eradicate these problems are needed. The following paragraphs present a review of existing sanitation systems with a latter focus on the system studied in this dissertation project, the cocopeat biofilter in Southeast Asia.

2.7 Wastewater issues in Vietnam

In Vietnam, a variety of wastewater management systems are employed. In densely populated areas, such as Ho Chi Minh City or Hanoi, typical flush toilets are used, and wastewater is collected in a traditional distribution system where it is either sent to a municipal wastewater treatment plant or is disposed, untreated, into a local water body. In less populated areas, one common technology is referred to as a "hello toilet" or simply "fish ponds". Small shacks, where defecation occurs, are suspended over fish ponds. The fish are sustained by consuming the waste products. This technology is very popular in the Mekong Delta of Vietnam, the test bed for my dissertation project. A survey taken showed that over 64% of the households utilized fish ponds for their wastewater technology. These ponds can cause significant harm to not only surrounding ecosystems due to nitrogen and phosphorus leaching, but they also create drinking water problems, such as the spread of typhoid fever, shigellosis and hepatitis E (Shaw, 2012).

Another common wastewater technology in the Mekong Delta is septic tanks. However, in general, they do not treat the water to appropriate discharge standards. One study analyzed pH, dry matter, somatic coliphages, male-specific bacteriophages, *E. coli*, *Salmonella* spp., *Enterococcus* spp. and helminth ova. Results showed that even after

many years of storage in the septic tank, viable phages and bacteria were accumulated in sludge. After application to land, the sludge-borne bacteria and helminth ova can persist for years (Herbst, 2009). Note that in many places, septic tank sludge gets transported off-site for further treatment. This increased handling of sludge presents exposure issues, and there are also technical, economic and institutional challenges with managing on-site sanitation systems such as septic tanks. Thus, there is a need for appropriate, low-cost and sustainable wastewater treatment technologies to remedy these issues.

2.8 Common sanitation technologies

Pit Latrine. The most commonly used sanitation technology in low-income countries is pit latrines. There are simple pit latrines and improved pit latrines which are modified with ventilation, a second underground storage, or a fly screen. A diagram of an improved pit latrine can be seen in Figure 2.2.

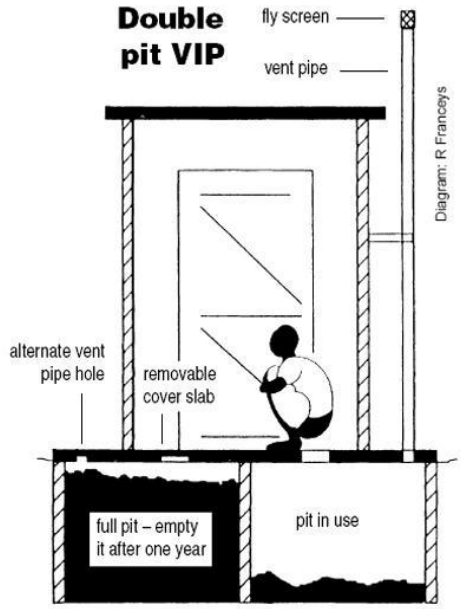


Figure 2.2 Improved Pit Latrine (Tearfund, 2012)

In these systems, excreta decompose into three states: gas, liquid and solid. The gases are released into the atmosphere or absorbed by soil, the liquids seep into the soil, and the solid residue accumulates in the underground storage. When the latrine is full, about half a meter from the top, it is usually back filled with soil and another pit latrine is constructed. The old latrine can be emptied by hand or truck, but is often left dormant. When emptied by hand, there are large health risks involved, as fresh excreta carries harmful pathogens, could become infected with worm, and flies could increase risk of infection. If the latrine is not lined, it may collapse when emptied (Running Water, 1999).

Biogas digesters. Biogas digesters (Figure 2.3) are concentrated in the developing world, where 5 million household digesters are in operation in China and India alone

(Lansing et al, 2008), and they are used throughout Vietnam, mostly in rural areas, for the treatment of household and agricultural wastewaters (An et al, 1997). They were introduced as a low-cost alternative source of energy to address the problem of acute energy shortages for households (An et al, 1997). This technology utilizes anaerobic digestion, the biological process of the breakdown of organic waste in the absence of oxygen. The microbes responsible for this process produce methane as a byproduct of degradation (Karakashev et al, 2005), which is often harnessed and utilized for household cooking (Lansing et al, 2008). These anaerobic digesters have been shown to reduce carbonaceous oxygen demand (COD) and increase the concentration of nutrients, such as ammonium, making the effluent from biogas digesters useful for fertilizer.

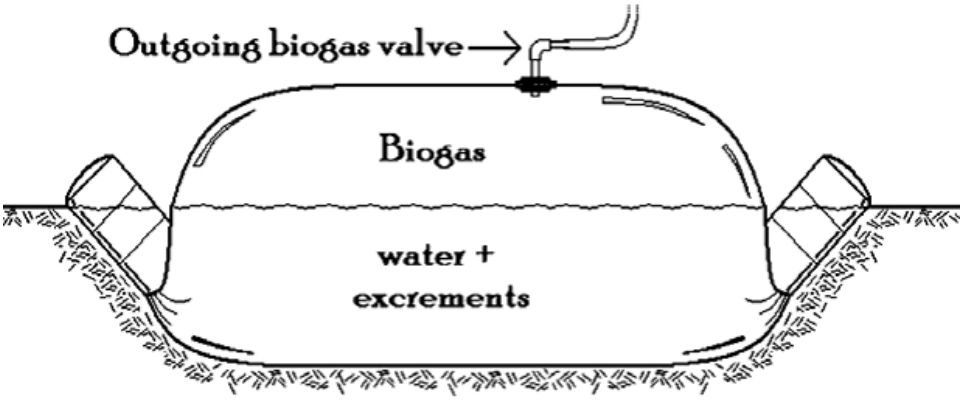


Figure 2.3 Simple biogas digester (Lansing et al, 2008).

Composting. Another practice, which is much less common, is to compost human waste. One way this is done is to place a household's solid waste in a container and to let it sit for a period of up to 3 months. During this time, the solids are broken down (i.e., degraded) by microorganisms. The resulting product is essentially a compost mixture which is rich in nutrients and is commonly utilized as fertilizer on agricultural fields. While this is a great means of "recycling" and can help maximize resources, in practice, it is often unsafe as many pathogens including *Salmonella* spp. are not eradicated. There are very few control methods and often safety protocols such as protective measures, crop restriction, application measures, human exposure control, and partial treatment mechanisms are lacking (Running Water, 1999).

2.9 Traditional peat filtration

Naturally occurring filtration media can be an effective and low-cost solution for sustainable wastewater treatment. They offer a benefit over artificial media such as foam, textiles and plastic because they utilize naturally occurring flora and fauna that are already locally available. However, often these natural media must be mined, which can have critical negative impacts on the environment and local ecosystems. Peat is one such material which can be utilized in fixed film systems, where the attached growth, colonization and reproduction of microorganisms are promoted for the treatment of wastewater streams (Sherman, 2006).

Sphagnum peat originates in bogs. As organic matter such as roots, stems, leaves, flowers, fruits and seeds decompose, they are saturated with water, preventing a complete degradation process. Below the layer of water, an anaerobic zone forms, slowing the degradation process all the more. In these conditions, partially decomposed plant material accumulates in layers and creates peat.

Sphagnum peat has been used for many decades to treat septic tank effluent. It's absorptive and odor controlling properties have been used for centuries, dating back to ancient times. It was also used in World War I as surgical dressings in field hospitals when cotton was scarce (Shaw, 2012). The first domestic on-site peat wastewater treatment system was set up in 1978 and it was shown that wastewater could be treated to drinking water standards (Shaw 2012). Since then, many filters treating septic tank effluent from single family homes have been installed throughout the United States.

Peat filtration uses three main mechanisms to treat wastewater: physical filtration, absorption and microbial activity. Peat has been shown to have an adsorption capacity two to three times lower than activated carbon. It is effective in removing phenol, oil, and odorous gases such as dimethylamine, ammonia and hydrogen sulfide from industrial waste streams. It also has shown to be effective at removing antimony, copper, cadmium, lead, mercury, nickel, uranium, zinc, and zirconium (Viraraghavan, 1987). Peat has strong absorptive properties due to the high ion exchange capacity (Shaw, 2012). It is composed of mostly lignin and cellulose, but also contains polar functional groups such as alcohols, aldehydes, ketones, acids, phenolic hydroxides and ethers. Because of this, peat is positively charged and is very polar. Peat is especially effective at adsorbing

dissolved solids such as transition metals and polar organic molecules (Viraraghavan, 1987). Peat is also highly acidic (Shaw, 2012). The microbial activity mainly consists of diverse aerobic and facultative aerobic heterotrophic bacteria which are typically found in the upper portions. The lower portions of the filter have higher concentrations of nitrifiers. Consequently, wastewater is digested and absorbed in the upper portions of the filter and undergoes nitrification in the lower portions (Gustafson, 2002).

Peat systems have been utilized in the United States when traditional wastewater treatment is not available. They are especially ideal for:

- Undersized lots because they have a small footprint;
- Impermeable soils because instead of septic tank water being released to an unsuccessful drainfield, the water can be treated through the peat;
- Lots near surface water sources because septic tank effluent may contaminate local water sources if released to a drain field; and
- When high treatment levels are needed (Shaw, 2012).

Peat bioreactors are a passive system and require little to no maintenance. Traditionally, peat biofilters are operated under aerobic conditions (Shaw, 2012). However, previous studies have shown that their operation can be optimized to develop regions with varying redox potential. In my dissertation project, I will optimize aerobic and anaerobic zones within the filter to promote nitrification and denitrification. Key parameters of interest are hydraulic retention time, removal efficiencies, and the organic

and hydraulic loading capacities (Kennedy, 2001). Water content is also a critical parameter (Zhang, 2007). The EPA recommends a loading rate of 2 - 5 gallons per square foot per day. However, for a peat filter system, it has been recommended that the loading rate is 1 gallon per square foot per day (Gustofson, 2002).

In laboratory studies where peat is used to treat septic tank effluent, 95% BOD removal is achieved, along with 90% suspended solids and 80% COD. It was noted that the COD removal was lower than expected because, organic matter leached from peat itself (Vararaghavan, 1987). Peat liners have also been used in pit latrines as protection against the movement of phosphorus and bacteria. These liners did not affect the movement of nitrogen (Vararaghavan, 1987).

In the 1970s, peat-filled ditches were used to treat primary effluent in Finland. They were able to achieve >85% reduction in BOD and >99% reduction in coliform bacteria. Also, peat-sand filter beds have been successful at achieving a 95% reduction in BOD and 99% reduction in coliforms when secondary effluent is applied via spray irrigation. These systems were also able to reduce phosphorus from 7 to 0.5 mg/L. In another study with a sand-peat bed, it was noted that 90% of the wastewater nitrogen was removed via the system during the second and third year of operation, but during the fifth year only 50% was removed. This was attributed to the oxidation of the peat and release of nitrogen (Vararaghavan, 1987).

When full-size peat reactors were used in the field to treat septic tank effluent, 99% fecal coliform, 90% BOD and 90% COD removal was achieved. Phosphate reduction was lower, varying between 58 and 96% (Vararaghavan, 1987). In general, peat

beds have proved to have low efficacies at removing phosphorus from wastewater streams. This is particularly a problem if protecting nearby water sources is a priority. It has been shown that preformed rust and untreated steel wool can be added to the bed to increase phosphorus sorption, although information on the mechanisms of removal was not provided (James, 1992). This is likely a result of the lack of anaerobic treatment zone which is needed for phosphorous removal pointing to the need for system optimization.

In general, sphagnum peat has proved to be an effective packing medium for biofiltration wastewater treatment. However, the mining of sphagnum peat often has negative effects on the environment. Sphagnum peat bogs often contain rare plant and animal species and are harmed by mechanical extraction. Furthermore, the peat forms at very slow rates, on average 1 mm / year, preventing sphagnum peat from being an ideal renewable resource (Gustafson, 2002).

2.10 Cocopeat

Cocopeat is an abundant waste product in several countries in Southeast Asia, including but not limited to, Vietnam, Indonesia and the Philippines. It is a by-product of coconut processing plants and comes from the outer husk of the coconut (Figure 2.5). The coconut shell is shredded and then the fibers are removed. It contains approximately 30% fibers and the remaining 70% is ground pith, which has a soil-like texture. Unlike sphagnum peat, cocopeat does not need to be mined. As with other peat, cocopeat will degrade over time into a “muck-like” material (Gustofson, 2002). However, this mucky peat can then be processed and used for land application.

In the original study where cocopeat was first explored as a treatment support medium, it was thought that the microorganisms in the filter needed to only be exposed to aerobic conditions (Gustofson, 2002). However, in this dissertation study, we will experiment with aerobic, anoxic and anaerobic zones to encourage nitrification and denitrification as well as phosphorous removal.

Figure 2.4 Cross-section of coconut

2.11 Constructed wetlands

Constructed wetlands are man-made ecosystems involving natural materials (soil, plants, insects, etc.) that water is filtered through for the purpose of treatment. Typical packing media include water, gravel or soil. Photosynthetic plants (macrophytes) are in integral part of this wastewater treatment system. Wetlands treatment is successful because of the benefits of the macrophytes due to:

1. The leaves and stems above the water:

a. shielding from sunlight and reduce algal blooms,
b. insulate water from heat loss

2. Stems and roots:
    a. are colonized by bacteria and biofilms, accumulating a large bacterial population,
    b. catch colloids,
    c. may give off oxygen gas during photosynthesis, stimulating bacterial metabolism.

Wetlands are effective at removing nutrients, biological oxygen demand (BOD), and suspended solids (SS). Heterotrophic bacteria are primarily responsible for BOD removal. Nutrient removal is a combination of microbial processes and the macrophytes, due to incorporation of nitrogen and phosphorus into the plant mass. The majority of removal of nitrogen is most likely due to microbial nitrification and denitrification (Rittman and McCarty, 2001).

2.12 Nitrification

Nitrification is the microbiological oxidation of ammonium to nitrite to nitrate. Ammonium removal is a mandated process for some wastewaters due to its high oxygen demand (up to 4.57 g O_2/g NH_4^+-N) and toxicity to aquatic macroorganisms. Nitrifying bacteria are autotrophs (must fix and reduce inorganic carbon), chemolithotrophs (use inorganic chemicals for energy), and obligate aerobes (require oxygen for respiration).

Nitrifiers are slow growers, having low yield, small maximum specific growth rates, and large minimum sludge retention times in wastewater treatment processes, such as in the activated sludge process. Nitrification is a two-step process:

$$\frac{1}{6}NH_4 + \frac{1}{4}O_2 = \frac{1}{6}NO_2^- + \frac{1}{3}H^+ + \frac{1}{6}H_2O \qquad \text{Equation 2.1}$$

$$\frac{1}{2}NO_2^- + \frac{1}{4}O_2 = \frac{1}{2}NO_3^- \qquad \text{Equation 2.2}$$

First, ammonium is oxidized to nitrite by ammonium-oxidizing nitrifying microorganisms including *Nitrosomonas, Nitrosococcus, Nitrospira, Nitrosovibrio,* and *Nitrosolobus* (Equation 2.1). Second, nitrite is oxidized to nitrate by nitrite-oxidizing nitrifying microorganisms, *including Nitrobacter, Nitrospira, Nitrospina, Nitrococcus,* and *Nitrocystis* (Equation 2.2). Nitrifiers are sensitive to inhibition from unionized ammonia (NH_3; at higher pH), undissociated HNO_2 (low pH), anionic surfactants, heavy metals, chlorinated organic chemicals, and low pH. Nitrifiers must also compete with heterotrophic bacteria for dissolved oxygen and space. Their slow growth rate puts them at a specific disadvantage (Rittman and McCarty, 2001).

2.13 Denitrification

Denitrification is the reduction of nitrate or nitrite to nitrogen gas. In environmental applications, denitrification is applied when complete nitrogen removal is required, such as treatment of drinking waters with elevated levels of nitrite and nitrate,

or wastewater which will be discharged to waters that need protection against eutrophication. In order for denitrification to occur, the nitrogen must be in its oxidized form of either NO_3^- or NO_2^-. Denitrification and nitrification are often coupled as many wastewaters contain the reduced forms of oxygen. Denitrification can be carried out by heterotrophic, autotrophic, gram-positive, or gram-negative bacteria, including *Pseudomonas, Alcaligenes, Paracoccus, Thiobacillus,* and *Bacillus*. All denitrifiers are facultative aerobes, meaning they shift from oxygen respiration to nitrate or nitrite respiration when oxygen is limited. Therefore, in order to promote denitrification, oxygen must be limited. Denitrification occurs in a step-wise manner where nitrate is sequentially reduced to nitrite, nitric oxide, nitrous oxide, and nitrogen gas (equations 2.3, 2.4, 2.5, and 2.6; Rittman and McCarty, 2001).

$$NO_3^- + 2e^- + 2H^+ = NO_2^- + H_2O \qquad \text{Equation 2.3}$$

$$NO_2^- + e^- + 2H^+ = NO + H_2O \qquad \text{Equation 2.4}$$

$$2\,NO + 2e^- + 2H^+ = N_2O + H_2O \qquad \text{Equation 2.5}$$

$$N_2O + 2e^- + 2H^+ = N_2(g) + H_2O \qquad \text{Equation 2.6}$$

2.14 Biological phosphorus removal

Phosphorus removal can occur in wastewater treatment prior to biological treatment, during biological treatment, and after biological treatment. When phosphorus removal occurs outside of biological treatment, it is almost always carried out by chemical precipitation. In this dissertation, we focus on biological treatment of phosphorus, which can occur by normal phosphorus uptake into biomass and enhanced biological phosphorus uptake into biomass. Normal phosphorus uptake occurs naturally as phosphorus makes up 2 – 3% of biomass dry weight. The stoichiometric formula for biomass is $C_5H_7O_2NP_{0.01}$.

Enhanced phosphorus uptake by microorganisms occurs when certain heterotrophic bacteria sequester high levels of phosphorus as intracellular polyphosphate as an energy storage material. When this process is successful, the biomass contains 2 to 5 times the phosphorus content of normal biomass. In order to promote this process, alternating anaerobic and aerobic zones is required in order to allow time for the phosphate accumulating organisms to grow, but to also starve them of nitrate and oxygen, so that conditions select for bacteria that can store phosphorus as energy to drive the breakdown of other chemicals for electron acceptors (Rittman and McCarty, 2001).

Chapter 3. A novel method for field detection of *Vibrio cholerae* using membrane filtration technique to evaluate biosand filter performance in the Artibonite Valley, Haiti

3.1 Introduction

Over 2 billion people have gained access to improved drinking water sources since 1990 (WHO, 2014). Although it has been declared that the world has met the Millennium Development Goal (MDG) target for drinking water, there are still 783 million people in the world that do not use an improved drinking water source (WHO, 2014). This is especially critical for people whose drinking water sources are directly exposed to pathogens, such as those living in Haiti.

*Vibrio cholera*e is now ubiquitous in Haiti (Enserink, 2010). The cholera outbreak in Haiti began in late October 2010, just 9 months after a 7.0 earthquake struck the already fragile country (Walton, 2011). Over 93,000 people have been sickened by the epidemic and over 2,100 have died (Chin, 2011). This may be, in part, due to the lack of access to improved water sources. Only 51% of the rural population has access to an improved water source, which could be a household connection, public standpipe, borehole, protected well or spring, or rainwater collection (World Bank, 2013). There are several alternative water treatment options available for point-of-use application, covering a range of unit processes, including coagulation, filtration, and disinfection. Several point-of-use-treatments have been investigated for the developing world. Chlorine, solar disinfection (SODIS), and pasteurization have been used for disinfection

(Tumwine, 2005; Ubomba, 2008; Clasen, 2008). *Moringa Oleifera* seeds have been used to promote coagulation and flocculation (Doerr, 2005; Ndabigengesere, 1998). Ceramic pots and biosand filters have also been used for filtration (Brown, 2007; Sobsey, 2008). While point-of-use treatment is not ideal due to variability of treatment efficiency between users, user behavior and acceptance, and limiting factors such as cost and availability, they do provide a means of water treatment when municipal water treatment is not available.

Municipal water treatment is not available in most geographical areas in Haiti. However, a large number of biosand filters have been installed in the country over the past decade, in part due to the efforts of the not for profit organization, Clean Water for Haiti. Biosand filters provided users with some assurance when the cholera epidemic began in 2010, however, the removal efficiency of *Vibrio cholerae*, the causative agent for cholera, in biosand filters have never been specifically investigated. Although biosand filters have been evaluated for removal efficiency of indicator organisms such as *Escherichia coli* (Elliot et al, 2008), it is unclear if indicator organisms such as coliform bacteria are appropriate as indicators for *V. cholerae*.

The World Health Organization recommends *E. coli* as the best fecal indicator (Tallon et al 2005). However, total and fecal coliform are commonly used as indicator organisms to predict the presence of other pathogenic bacteria. *E. coli* and fecal coliform are generally interchangeable (Guidelines, 2006), and fecal coliform are a sub-category of total coliform. Coliform are recognized as acceptable indicators of the efficacy of treatment and the disinfection process (WHO 2003, FPTCDW 2002), but are considered

unreliable indicators of fecal contamination (Tallon et al 2005). A correlation has never been established linking coliform and *V. cholerae* presence and it has been shown that indicator bacteria do not always accurately represent concentrations of specific pathogens of interest, nor do removal efficiencies necessarily indicate removal of pathogenic organisms. For example, in Elliot et al. (2008), biosand filters were seeded with *E. coli*, echovirus type 12, and bacteriophages (MS2 and PRD-1). Echovirus 12 reductions were comparable to *E. coli* reductions, but bacteriophage reductions were much lower and had a large range of removal, suggesting that virus reduction by the biosand filter may differ depending on the viral agent and that *E. coli* reductions do not necessarily indicate reduction efficiency. The same problem may be encountered for specific bacteria of interest, as bacteria have different surface proteins and charges which will may lead to different attachment efficiencies during biofiltration (Stoodley, 2002; Mueller, 2007; Gallardo-Moreno, 2003).The surface characteristics of a specific bacterium, such as hydrophobicity or surface tension, is believed to be the determinant factors for initial interaction with a surface (Gallardo-Moreno), such as sand. Biofilm formation offers *V. cholerae* a specific advantage as it can exhibit a diverse range of phenotypes on surfaces (Mueller, 2007).

Because indicator organisms such as *E. coli*, total coliform, and fecal coliform may not be appropriate indicators for pathogens of interest, there is a need for the development of field based methods to quantify pathogens. In particular, most efforts have focused on coliform with little work on other species. This is problematic as a

correlation does not always exist between removal efficiencies of indicator bacteria and the pathogenic target. Thus, there is a clear need for better field methods.

There are a few methods available for *V. cholerae* detection in the field, such as dipstick tests, cholera SMART kits, and polymerase chain reaction (PCR). Dipstick tests are a relatively cheap, simple, and fast way to detect presence or absence of *V. cholerae* and they can be used to test a large number of samples in a short time (Nato et al, 2003). SMART kits have been reported to give a presence or absence indicator within 15 minutes testing the sample (Hasan, 1993). However, neither of these tests gives a measure of *V. cholera* concentration in a given water sample. PCR is a useful tool for the detection of *V. cholerae*, as genetic probes can allow for the targeting of species, serogroups, and toxins in any given sample. However, PCR results are generally reported as presence/absence, and they cannot provide direct evidence of cell viability (Lipp et al, 2003).

In the present study, a plate count test utilizing membrane filtration technique was developed which could be applied for the measurement of viable *V. cholerae* cell concentration in the field. The method is compared to traditional coliform plate methods to determine if coliform are an appropriate indicator of *V. cholerae* concentration in source waters and *V. cholerae* removal efficiency in field biosand filters. To this end, 50 biosand filters in the Artibonite Valley of Haiti, the epicenter of the cholera epidemic, were assessed for removal efficiency of *V. cholerae* and total coliform. Schmutzdecke samples were collected from each filter to investigate the correlation between removal

efficiency and schmutzdecke composition, specifically biofilm microbial community and extracellular polymeric substance (EPS). Parameters controlling removal were also analyzed.

3.2 Materials and Methods

Bacterial strain and growth conditions. V. cholerae 0395 was obtained from the Kuehn lab at Duke University. Cells from a single colony were grown in Luria-Bertani (LB) broth (10 g NaCl, 5 g yeast extract, 10 g tryptone per liter) to late log phase at 37 ^{o}C. Frozen stocks of *V. cholerae* were made by mixing 1 mL of late log phase *V. cholerae* in LB broth mixed with 1 mL of glycerol and stored at -80 ^{o}C. Each experiment was started from -80 ^{o}C glycerol stocks, where pure frozen stock was streaked onto an LB plate and incubated overnight at 37 ^{o}C. Cells from a single colony were grown in LB broth and were then used in downstream processing. New cultures were used for each experiment. Pure culture stocks were routinely plated to verify community purity.

Total coliform was isolated by filtering activated sludge from the North Durham Wastewater Treatment Plant with a 0.45 µm mixed cellulose filter (Whatman, Piscataway, NJ) and placing the filter on M-Endo agar (Hi media, San Francisco, CA) according to Standard Method 9222B (Standard Methods, 2012). Briefly, M-Endo agar was prepared by suspending 51 g of M-Endo agar in 1 L of deionized water with 20 mL of non-denatured ethanol. The mixture was heated to boiling and allowed to cool to 60 ^{o}C before growth medium was added into each Petri dish. Wastewater samples were serially diluted in PBS buffer and filtered through 0.45 µm mixed cellulose ether filters

(Whatman, Piscataway, NJ) using a vacuum pump. Filters were then placed on the prepared plates and incubated at 35 ºC for 24 h. A single colony with metallic phenotype typical of total coliform was enriched and cultured in LB broth overnight. The culture was maintained in glycerol at -80 ^{o}C as described above.

Total coliform and V. cholerae quantification. Membrane filtration was selected as the basis for our method development for the field quantification of bacteria because of the ease of implementation in the field and its relatively low cost. To this end, bacteria specific media were selected for total coliform (M-Endo medium) and *V. cholerae* (thiosulfate citrate biles salts-sucrose-(TBCS) medium). To minimize field equipment requirements, the standard method for membrane filtration was modified and tested in the lab.

Briefly, sufficient powdered growth medium to make 40 mL of M-Endo broth was initially aliquoted into sterile 50 mL plastic tubes. Deionized water was filtered through 0.2 µm mixed cellulose ester filters (Millipore, Billerica, MA) and added to the tubes to a final volume of 40 mL. Plates were prepared by pipetting 2 mL of broth onto a pad. For enumeration, water samples were filtered through 0.45 µm mixed cellulose ether filters (Whatman, Piscataway, NJ) using a vacuum pump. Filters were then placed on the medium-soaked pad and plates were incubated at 35 ºC for 24 h. As shown in Figure 3.1a, total coliform can easily be differentiated from other bacteria based on their unique phenotype (i.e., they develop a metallic sheen when grown on M-Endo medium). For the *V. cholerae* quantification, the same method was used except that TCBS (Hi media, San

Francisco, CA) was substituted for the M-Endo medium. Using this method, *V. cholerae* can easily be differentiated from other bacteria as it expresses a yellow phenotype (Figure 3.1b).

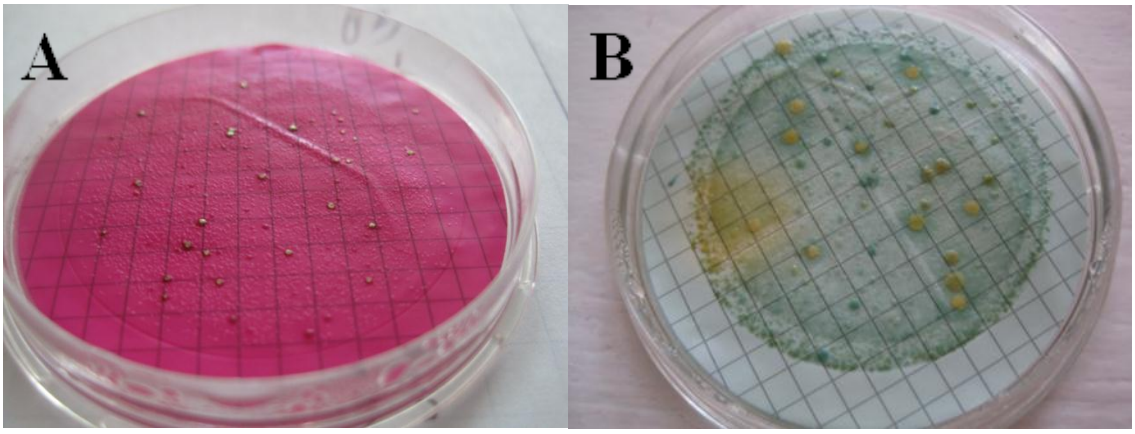


Figure 3.1 Mixed culture from field study using described methods plated on A) M-Endo broth (total coliform shown as metallic colony), and B) TCBS broth (*V. cholerae* shown as yellow colony)

To confirm method accuracy and develop a standard curve, results from these plate methods were compared to hemocytometer bacterial cell counts. Hemocytometer quantification allows for the direct microscope count of bacteria suspended in water or media. Hemocytometer quantification was performed from frozen bacterial stocks subsequently transferred to LB plates. LB liquid medium was inoculated with cells. The pure cultures were grown with continuous agitation at 250 rpm at 37 ºC overnight. Bacterial pellets were obtained by centrifugation, washed and resuspended in PBS buffer. Serial dilutions were performed in PBS buffer. For each dilution, an optical density (OD) reading was taken at 600 nm on a Hach DR/2500 spectrophotometer (Loveland, CO) and hemocytometer counts were performed to generate a standard curve which relates optical

density reading to concentration of cells. Hemocytometer counts and membrane filtration technique plate counts were compared to determine if there was a linear relationship between resulting cell quantifications of the two methods.

Field water sampling. Fifty biosand filters were tested in 5 different villages in the Artibonite Valley, the epicenter of the cholera epidemic (Fraser, 2010). Sample villages were chosen based on how long biosand filters were in operation (i.e., either 0 days, 4 days, 1 month, 3 months, or 1 year). Note that because of this, village effects cannot be separated from age of filter. Within each village, there was a range of water sources, including shallow, hand dug wells, deep wells, and water from the Artibonite River. Upon sampling at each home, the time, age of filter, typical use, last time filter was used, and source were noted. Although frequency of use and consistency of use could not be accurately measured during this field study, and TOC concentrations were not measured, water sources were annotated. Three samples of the schmutzdecke (4" depth, 0.25" diameter) were collected using a small tube and were placed into sterile 1.5 mL microfuge tubes (VWR, Radnor, PA). Also, samples of influent and effluent water were collected in triplicate. Six L of influent and effluent water were collected gravimetrically, stored in sterile whirlpak bags (Nasco, Fort Atkinson, WI) and kept on ice until being stored at 4 ºC overnight prior to enumeration. Effluent water was collected directly from the spout of the biosand filter. Input water was collected from the source water collection bucket with a plastic cup.

Figure 3.2 Map of Haiti with the source of the cholera epidemic (★) and the area sampled (▭).

Field bacterial removal efficiency measurements. Total coliform and *V. cholerae* concentrations were measured by the membrane filter technique as described earlier. For each sample, three dilutions were quantified to ensure appropriate enumeration. To this end, 3 different volumes (300, 30, and 10 mL) of each water sample were vacuum filtered through 0.45 µm mixed cellulose ester filters (Whatman, Piscataway, NJ) in triplicate. The filters were then placed on Petri dishes containing absorbent pads (Pall Life Science, Ann Arbor, MI) which were previously treated with either TCBS or m-Endo growth

media for *V. cholerae* and total coliform, respectively. The Petri dishes were then incubated at 35 °C for 24 h. Total coliform and *V. cholerae* quantification on both the influent and effluent water samples were performed in triplicate. All equipment was sterilized with ethanol between each filter sample set. Negative controls were performed without water by performing the same procedure as with regular sample tests. The filter was opened, removed with sterilized tweezers, placed on the filtration device, the pump turned on and then off after several seconds, the filter removed with tweezers, placed onto the respective plate, and incubated at 35 ºC.

DNA isolation. DNA was extracted from schmutzdecke samples using phenol:chloroform DNA extraction. In brief, cells were resuspended in TE buffer, washed, and resuspended in TE buffer, 10% SDS and Proteinase K. To lyse cells and isolate DNA, cells were vortexed and incubated at 56 °C for 2 h. To purify the DNA, phenol/chloroform extraction was performed twice by adding an equal volume of phenol/chloroform, vortexing gently, centrifuging at 13,000 rpm for 1 min, and removing the aqueous phase and placing in a clean tube. Then, an equal volume of chloroform was added, gently vortexed, centrifuged, and the aqueous phase was placed in a clean tube.

To precipitate the DNA, ethanol precipitation was performed. One M NaCl and ice cold 190 proof ethanol was added, the mixture was mixed by inversion, and incubated in the freezer at -20 ^{o}C overnight. Then, samples were centrifuged at 13,000 rpm for 10 min to form a pellet of DNA. The ethanol mixture was decanted and ice cold 70%

ethanol was added and centrifuged to desalt the pellet. The supernatant was removed and once the liquid evaporated, DNA was resuspended in Tris-Cl.

The phenol/chloroform/isoamyl alcohol mixture was obtained from Life Technologies (Carlsbad, CA). Concentration and purity of DNA was measured on a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Community analysis by T-RFLP. PCR was performed, targeting the *16S* gene, with the 6 – carboxyfluorescein-labeled fluorescent forward primer (27F) and reverse primer 1392R. PCR conditions used were 94 ºC for 5 min followed by 35 cycles of 30 s at 94 ºC, 30 s at 50 ºC, and 30 s at 72 ºC, with a dissociation step at the end for quality control. Amplicons were purified utilizing a Qiagen PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Final PCR product concentrations and purity were measured on a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). One hundred ng of purified PCR product and 10 U of *Msp1* (New England Biolabs, Beverly, MA, USA) were used for each T-RFLP reaction. The mixture was incubated at 37ºC for 2 h. Analysis of fragments was performed using an Applied Biosystems 3100 capillary sequencer (Foster City, CA) with POP6 polymer and ROX-labeled MapMarker 1000 size standards (Bioventures, Inc., Murfreesboro, TN) at the Duke University DNA Analysis Facility (Durham, NC). Standard procedures were followed.

In order to perform principal component analysis (PCA) and non-metric multidimensional scaling (nm-MDS), T-RFLP profiles were visualized using Applied

Biosystems GeneScan v3.7.1 software (Foster City, CA). T-REX online (Culman et al., 2009) software was used to process raw data through T-RF alignment to look at presence/absence of fragments. Any fragment 50 bp or smaller was excluded from data set to ensure no primer dimers were included in the analysis. Ordination plots were analyzed for group clustering using the Paleontological Statistics software (PAST) statistical software to determine if ordination clusters were statistically similar (Hammer, Ø. & Harper, D.A.T. 2006. Paleontological Data Analysis. Blackwell.).

EPS extraction and analysis. EPS was extracted using a cation exchange resin as previously described (Frolund et al, 1996; Badireddy et al, 2011). Briefly, 75 mg of the Na^+ form of a polystyrene divinylbenzene microporous ion exchange resin (Dowex 50WX8, 20e50 mesh, Sigma Aldrich)/g volatile suspended solids (VSS) was added to 50 mL of sample and shaken at 900 rpm for 4 h at 4 ^oC. A two-step centrifugation at 4 ^oC (first at 5000g for 15 min and then at 12,000g for 30 min) followed by filtration using a 0.45 mm cellulose acetate membrane was used to remove resin, microorganisms, and residual debris to obtain an EPS sample for further analysis. After collection from each biosand filter, samples were stored at -20 ^oC until further analysis.

Protein was quantified by the Modified Lowry Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin standards. Briefly, 40 µL of standard or sample was plated on a microplate (Thermo Fischer Scientific) in duplicate. Two hundred µL of Modified Lowry Reagent was added to each well, and the plate was shaken at 1500 rpm for 30 s using a Thermo Electron Corporation Multiskan MCC

(Waltham, MA) plate reader. Then 20 µL of prepated 1X Folin-Ciocalteu reagent was added, and the plate was shaken at 1500 rpm for 30 s. The plate was covered and incubated at room temperature for 30 min. Then the absorbance was recorded at 690 nm (Figure A1).

Carbohydrates were measured using the phenol-sulfuric acid method against glucose standards (Bariddy et al, 2011). Briefly, 10 µL of standard or sample was plated on a microplate in duplicate. Five µL of 80% phenol solution (w/v) was added to each well and the plate was shaken at 1500 rpm to mix. Then 200 µL of sulfuric acid was added to each well in a stream. The plate was incubated at room temperature for 10 min and the absorbance was recorded at 490 nm (Figure A2).

Uronic acid was determined as described in Bariddy et al (2011). Briefly, 40 µL of standard or sample was added to a microplate in duplicate. Two hundred µL of sulfuric acid (96% w/w) containing 120 mM sodium tetraborate was added. The plate was shaken at 1500 rpm to mix and then incubated at 80 ^{o}C for 1 h. Then 100 µL of m-hydroxydiphenol was added to each well, the plate was shaken at 1500 rpm, and incubated at room temperature for 15 min. The absorbance was measured at 540 µm (Figure A3).

Statistical Analysis. The unpaired, two tailed student's t test was used to identify statistical differences between samples. Results were considered statistically different when the p-value <0.05.

3.3 Results and Discussion

No statistical difference was detected ($p>0.05$) between membrane filtration technique and hemocytometer counts for either total coliform or *V. cholerae* using the described methods (Figure 3.3). A linear correlation was observed between *V. cholerae* concentration obtained via the described membrane filtration technique and hemocytometer counts, with a R^2 value equal to ~1. Additionally, there was a linear correlation between total coliform concentration obtained via the described membrane filtration technique and hemocytometer counts, with a R^2 value equal to ~1. These data suggest that the described membrane filtration technique methods, both for total coliform and *V. cholerae*, are accurate compared to concentrations obtained via microscope counts, and provide an accurate measure of cell concentration.

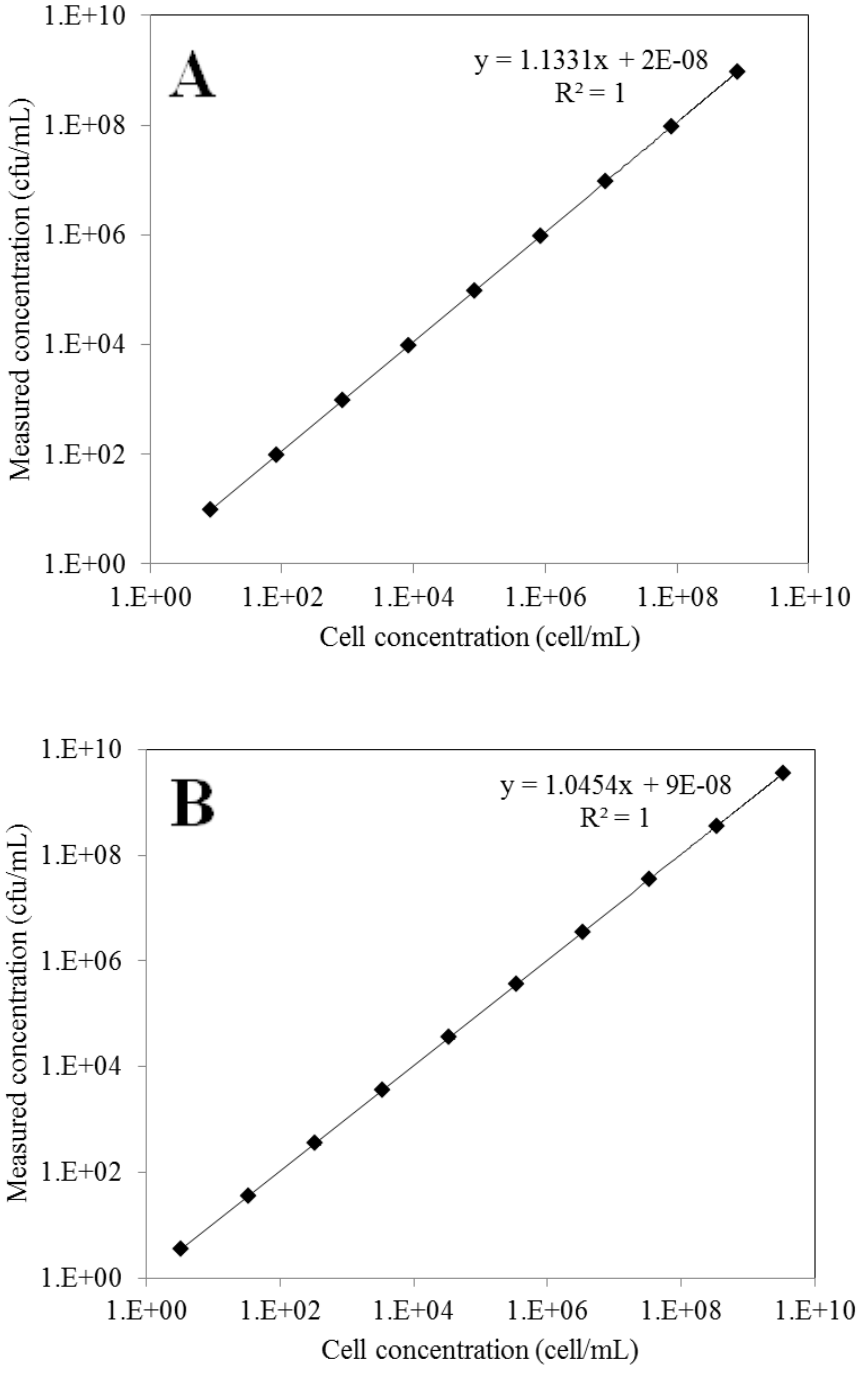


Figure 3.3 Experimental concentration (membrane filtration technique) vs. actual concentration (hemocytometer counts) of A) *V. cholerae*, and B) total coliform. Error bars represent the standard deviation of triplicate samples (not visible due to low standard deviations).

Total coliform as an indicator of V. cholerae in field biosand filters. Source water concentrations of total coliform ranged from approximately 100 – 2000 cfu/100 mL. Source water concentrations of *V. cholerae* ranged from approximately 100 – 3000 cfu/100 mL. Figure 3.4 shows concentration of *V. cholerae* versus total coliform from source waters tested in the Artibonite Valley, Haiti using the described membrane filtration technique methods. A 1:1 line is added for comparison. If total coliform were a perfect indicator of *V. cholerae* concentration, all points would be on the 1:1 line. As can be seen, in 90 % of the influent samples from the study, coliform underestimated *V. cholerae* concentration (n = 26). If *V. cholerae* is preferentially removed in biosand filters as compared to *V. cholerae*, the underestimation may not be critical, however more research is needed to determine if this is the case. However, when comparing treatment efficiency, total coliform removal efficiency underpredicted *V. cholerae* removal efficiency (n=16) (Figure 3.5). It can be seen that there is negative total coliform removal efficiency, indicated higher concentrations of total coliform in the effluent than in the influent. These data points are associated with filters that have been operating for four days. The sand within the filter may be contributing to the total coliform load, as during construction, the sand may not have been rinsed thoroughly enough to remove the naturally occurring microbial community of the sand, which would generally include coliform bacteria. These data suggest that total coliform removal efficiency is not a good predictor of *V. cholerae* removal efficiency, however, in this case, the coliform provide a conservative measurement. It is possible that schmutzdecke composition and

physical/chemical attachment play an important role in dictating which bacterial strains will be removed preferentially. Further discussion on this point is presented below.

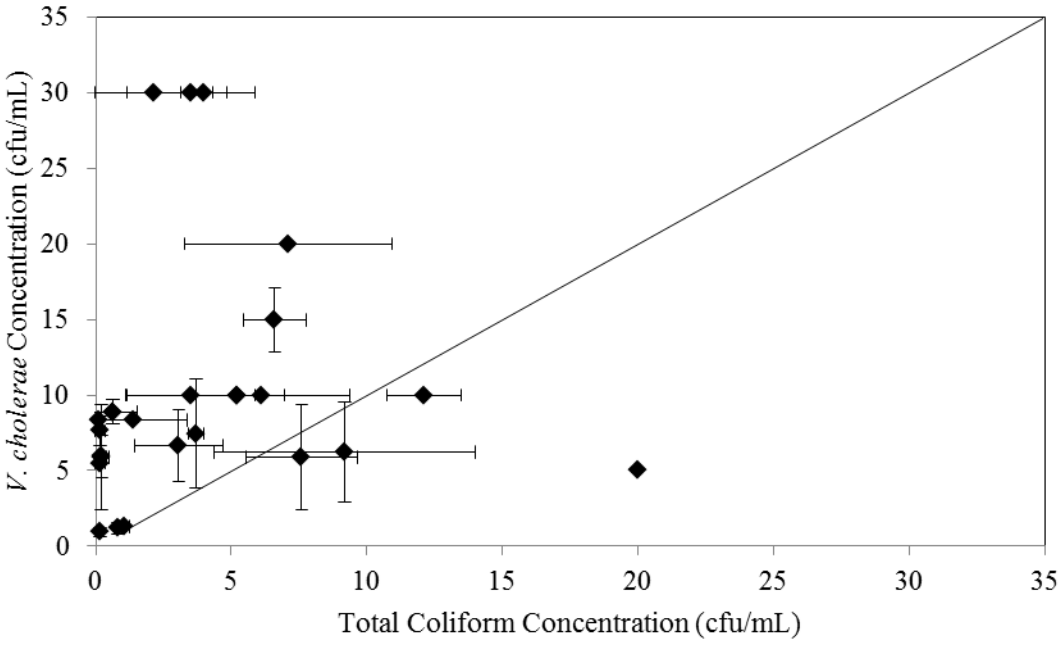


Figure 3.4 Total coliform concentration does not correlate well with *V. cholerae* concentration in influent waters. Error bars represent the standard deviation of triplicate samples. (n=26).

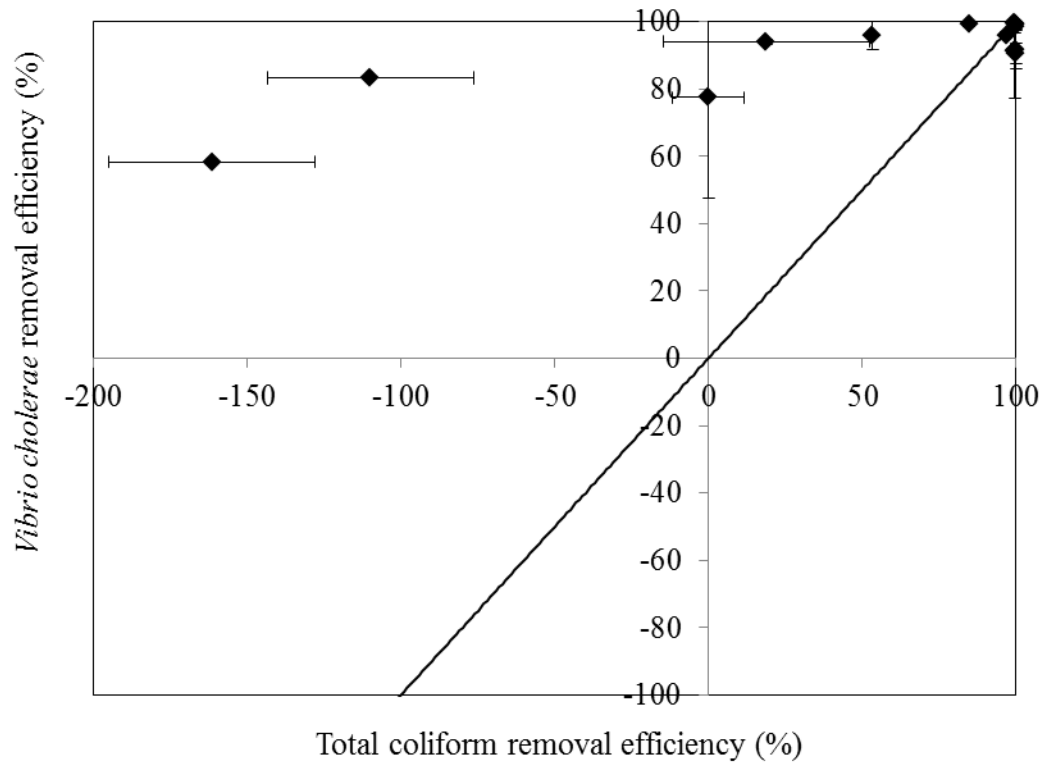


Figure 3.5 Total coliform removal efficiency is not a good predictor for *V. cholerae* removal efficiency in biosand filters. Error bars represent the standard deviation of triplicate samples. (n=16)

Time in operation affects removal efficiency of total coliform and V. cholerae in field biosand filters. The average removal of *V. cholerae* for all collected samples was 80 ± 31%. The average removal of total coliform was 63 ± 68%. However, when samples are separated into groups based on time in operation (village or filter age), standard deviations are minimized, suggesting that time in operation is an important parameter controlling removal over other variables including water source, idle time, and frequency of use (Figure 3.6). This is consistent with previous work as Elliot et al (2008) showed

that removal efficiency of *E. coli* increased as time in operation increased in a lab study. This was attributed to the formation of the biofilm.

The average percent removal for *V. cholerae* from four day old, 1 month, 3 months and 1 year filters were 65 $\pm$ 24% (n=10), 92 $\pm$ 8% (n=10), 96 $\pm$ 3% (n=9) and 97 $\pm$ 3% (n=10), respectively. As filter operation time increases, the removal efficiency of *V. cholerae* increases. This was attributed to the formation of the schmutzdecke (discussed below). Filters having operated for less than one month showed the largest range of removal efficiencies (from 19 to 94%). The range became much smaller with extended operation (77 to 99% for 1 month filters, 90 to 99% for 3 months filters, and 91 to 100% for 1 year filters). Beyond three months of operation, removal efficiency does not improve significantly ($p>0.05$), suggesting that the first three months of operation play a critical role for biofilm formation. The data also suggest that reactors reach steady state removal within the initial three months of operation independent of other variables. It should be noted that users are advised to charge six buckets of water before consuming the water effluent (Clean Water for Haiti, 2011), but this may not be sufficient to provide high level of pathogen removal. It may be more advisable to design guidelines that take other operational parameters into consideration to better target when the water is safe for consumption. As shown herein, in some instances some biosand filters were achieving *V. cholerae* removal in excess of 90% by the 1 month sampling point. However, it is unclear from our study what triggered the higher removal rates in some filters over others. A larger study should be carried out to further establish correlations between water characteristics, operational parameters and water safety in terms of pathogen removal.

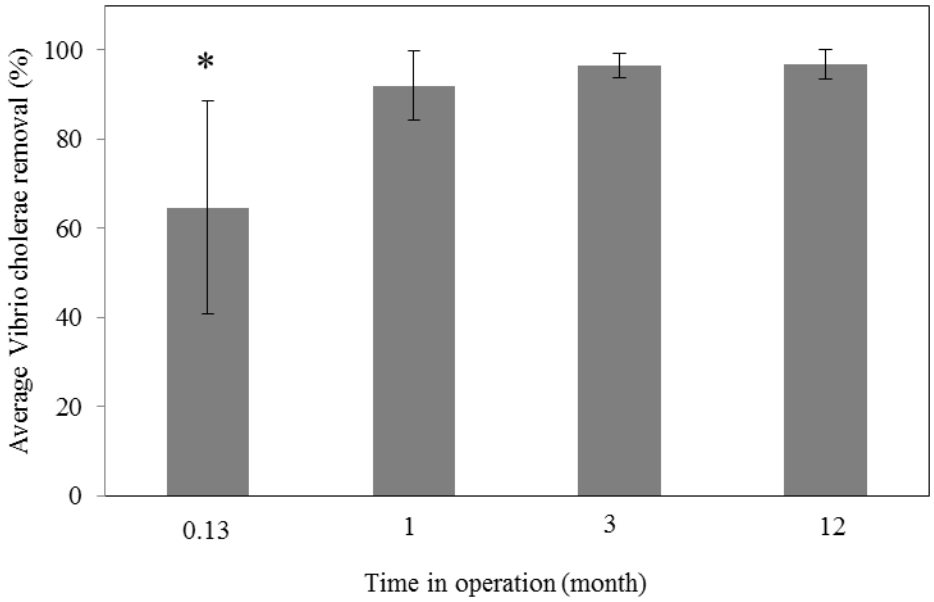


Figure 3.6 Average *Vibrio cholerae* removal efficiency (%) based on time in operation. Error bars represent the standard deviation within the age group. The * indicates statistical difference from filters having operated for 12 months.

Other key factors play a role in removal efficiency. Schmutzdecke microbial communities do not cluster as a function of time of operation (Figure 3.7). This result is not unexpected as microbial profiles will vary greatly based on source water characteristics, idle time, and relative amount of biofilm (indicated by amount of EPS). These factors are explored below.

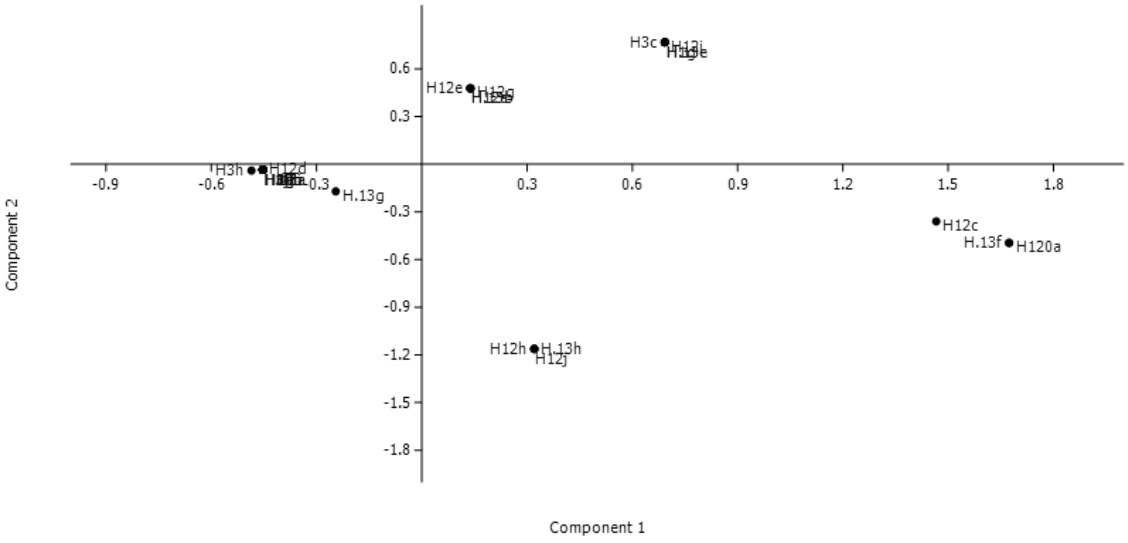


Figure 3.7 Principal component analysis (PCA) of Haiti schmutzdecke microbial community (T-RFLP).

Figure 3.8 shows a non-metric multidimensional scaling plot of biofilm communities with three environmental factors – idle time, time in operation, and amount of EPS. Time in operation accounted for much of the spread, indicated by the spread along that axis. Idle time and mg of EPS per g of VSS were inversely related, suggesting that these factors have opposite effects on the microbial community. This may be because the biofilms experiencing short idle times do not need as much EPS as bacteria are always present. However, the biofilms experiencing longer idle times may need more EPS because bacterial growth may be limited. Analyzing the nm-MDS plot with PAST, time in operation accounts for 47.5% of the microbial community differences while idle time accounts for 37.1% of the microbial community differences. These data suggest that

time in operation and idle time significantly affect the biofilm and, hence, filter performance.

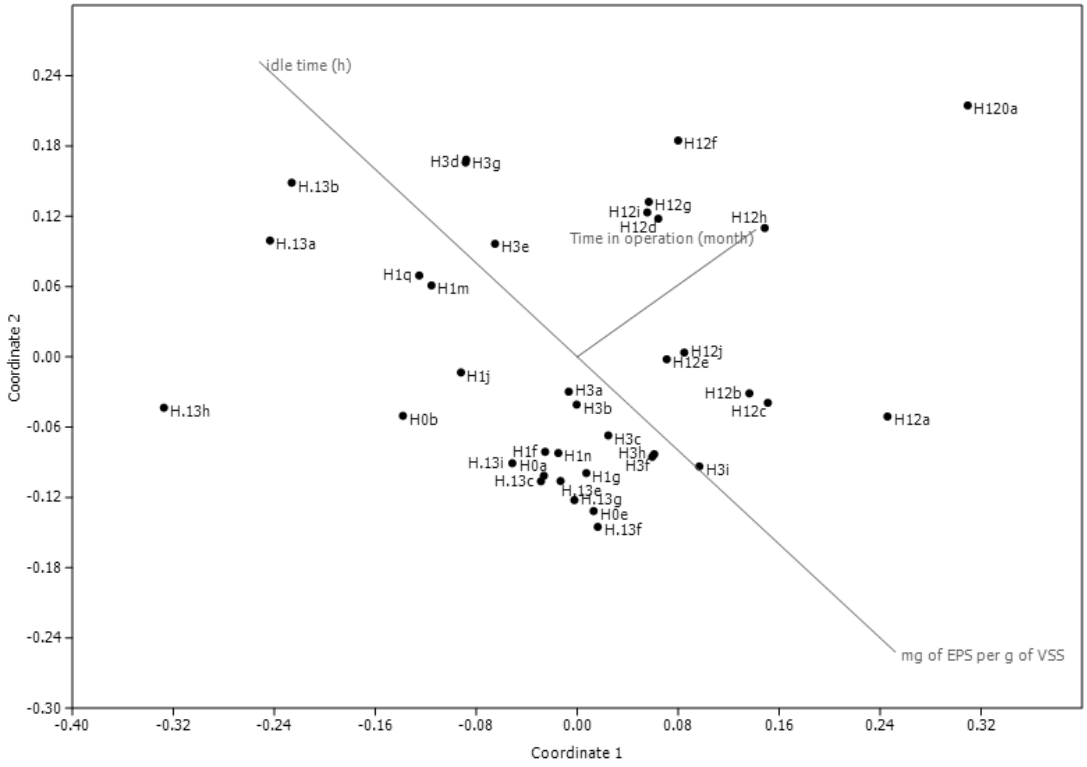


Figure 3.8 Non-metric multi-dimensional scaling (nm-MDS) plot of Haiti schmutzdecke microbial community (T-RFLP analysis) with three environmental factors (idle time, filter time in operation, and amount of EPS).

Interestingly, there is no clear correlation between time in operation and amount of EPS (Figure 3.9). These data suggest that there are other key parameters contributing to removal efficiency besides time in operation. One reason for this is that time in operation does not necessarily indicate schmutzdecke age. The standard protocol calls for

users to stir the schmutzdecke when the effluent flow rate becomes too slow for the user. When mixing occurs, some schmutzdecke detachment will occur thereby increasing the effluent flow rate. The schmutzdecke will then need to be reconstituted (i.e., grow) prior to the biosand filter reaching an optimal removal efficiency once more. It is likely that users with water sources containing higher TOC and higher turbidity will need to stir the schmutzdecke more frequently as compared to users with a deep well as a water source (corresponding to water with low TOC and low turbidity).

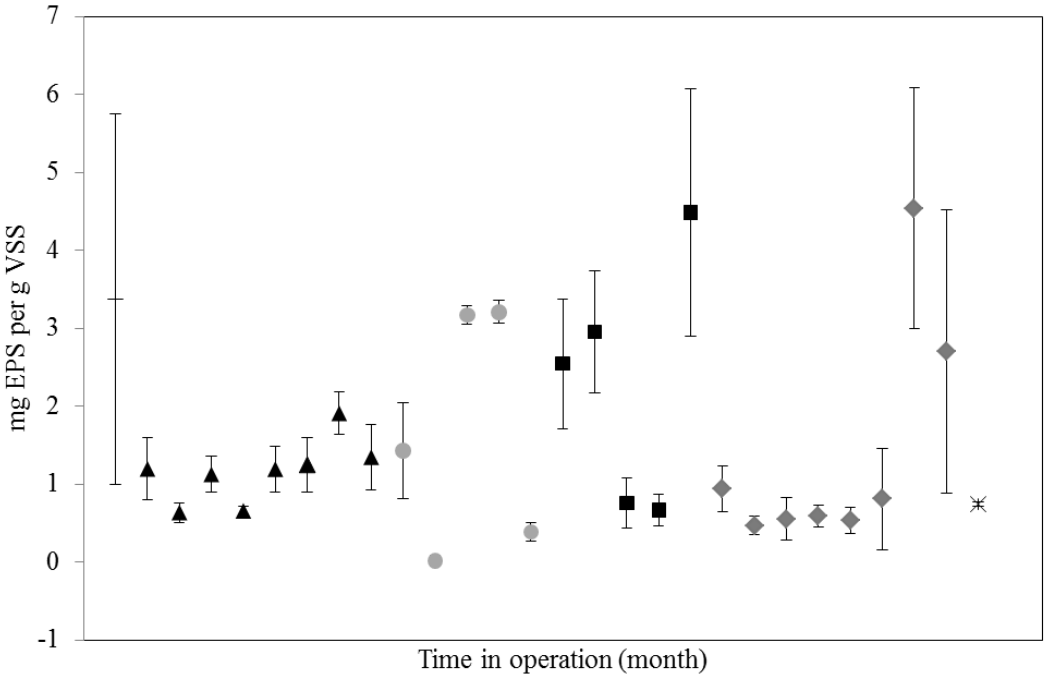


Figure 3.9 EPS in Haiti biosand filter schmutzdecke samples, filter time in operation of less than 1 day (+), 4 days (▲), 1 month (●), 3 months (■), 12 months (◆) and 120 months (x). Error bars represent the standard deviation of duplicate samples.

In addition to water source characteristics, other parameters including idle time, frequency of use, and consistency of use may play a critical role in treatment efficacy. As previously mentioned, idle time accounted for 37% of the microbial community differences between filters. If a filter experiences too long of an idle time (>48 h) or an inconsistent use, the biofilm may began to deteriorate due to starvation or lack of oxygen (Lawrence et al, 2004; Hunt et al, 2004; van der Kooij et al, 2003). If the biosand filter is used too often (i.e., more than twice per day), the residence time may not be long enough for a biofilm to develop or for other removal mechanisms to take place, such as starvation and predation (Elliot, 2008). Such frequent use may also lead to more biofilm shearing events, decreasing the amount of EPS. Although frequency of use and consistency of use could not be accurately measured during this field study, and TOC concentrations were not measured, water sources were annotated, and within each age group, users retrieved water from deep wells, shallow wells, canals, and the Artibonite River. We hypothesized that differences in source water characteristics, namely total organic carbon (TOC), may have accounted for some of the variability in amount of EPS and microbial community. This hypothesis is explored in Chapter 4 using laboratory data.

3.4 Conclusions

In this chapter, a method for the quantification of *V. cholerae* in the field was developed and implemented. The plate count method for the enumeration of *V. cholerae* in the field was shown to be accurate as compared to hemocytometer counts, yielding differences between the two methods that were not statistically significant. This test

offers a relatively inexpensive and simple way to visualize *V. cholerae* colonies in a mixed culture setting by using selective media (TCBS) which presents *V. cholerae* as yellow colonies. It was found that total coliform removal is not the same in a biosand filter as *V. cholerae*, suggesting a need for pathogen specific tests when evaluating biosand filters in an outbreak situation. In addition, key parameters were identified which control *V. cholerae* removal in field biosand filters, including: time in operation, amount of EPS, and idle time.

Chapter 4. Evaluating the Role of Total Organic Carbon in Predicting the Treatment Efficacy of Biosand Filters for the Removal of *Vibrio cholerae* in Drinking Water

4.1 Introduction

Over 2 billion people have gained access to improved drinking water sources since 1990 (WHO, 2014). Although it has been declared that the world has met the Millennium Development Goal (MDG) target for drinking water, there are still 783 million people in the world that do not use an improved drinking water source (WHO, 2014). This is especially critical for people at risk of exposure to deadly pathogens including *Vibrio cholerae, Shigella,* and *Salmonella*, such as those living in Haiti where *V. cholera*e is now ubiquitous (Enserink, 2010). Many biosand filters have been installed in Haiti, but it is unclear whether these filters have the capacity to effectively remove *V. cholerae* to a safe concentration for consumption. Several biosand filter studies have been performed investigating the treatment efficacy of thermotolerant coliforms or *Escherichia coli* in a lab (Duke et al 2006, Kaiser et al 2002, Elliot 2008, Stauber et al 2006, Baumgartner et al 2007) and field setting (Stauber 2009, Stauber 2006, Duke 2006, Tiwari et al 2009, Fiore et al 2010). However, there have been no studies investigating the efficacy of the biosand filter to remove specific pathogens of interest such as *V. cholerae*.

Furthermore, while the structure of the schmutzdecke (biofilm) following the initial construction of a biosand filter, as well as its properties following a filter's long

term operation, are known to be critical to the quality of the drinking water effluent, little research has been performed to characterize the schmutzdecke layer and determine what properties control the quality of water effluent.

In the previous chapter, we discussed parameters controlling schmutzdecke composition and hence, filter performance. These factors included: time in operation, idle time, and amount of EPS. However, we hypothesized that influent water characteristics may also affect filter performance. To test this hypothesis, three biosand filters were constructed and tested in a laboratory setting. In particular, parameters controlling *V. cholerae* removal efficacy were assessed. Each biosand filter was loaded with a different total organic carbon loading to simulate field conditions of varying TOC loadings between filters. *V. cholerae* pure cultures were charged intermittently to assess the effect of the number of charges on removal efficacy. Microbial community structure and EPS content of the schmutzdecke were also analyzed.

4.2 Methods and Materials

Bacterial strains and growth conditions. Bacterial strain and growth conditions. *V. cholerae* 0395 was obtained from the Kuehn lab at Duke University. Cells from a single colony were grown in LB broth (10 g NaCl, 5 g yeast extract, 10 g tryptone per liter) to late log phase at 37 ^{o}C. Frozen stocks of *V. cholerae* were made from 1 mL of late log phase *V. cholerae* in LB broth mixed with 1 mL of glycerol and stored at -80 ^{o}C. Each experiment was started from -80 ^{o}C glycerol stocks, where pure frozen stock was streaked onto an LB plate and incubated overnight at 37 ^{o}C. Cells from a single colony

were grown in LB broth and were then ready for downstream use. New pure cultures were grown for each experiment. Pure culture stocks were also plated to verify purity.

Total coliform was isolated by filtering activated sludge from the North Durham Wastewater Treatment Plant with a 0.45 µm mixed cellulose filter (Whatman, Piscataway, NJ) and placing the filter on M-Endo agar (Hi media, San Francisco, CA) according to Standard Method 9222B (Standard Methods, 2012). Briefly, M-Endo agar was prepared by suspending 51 g of M-Endo agar in 1L of deionized water with 20 mL of non-denatured ethanol. The mixture was heated to boiling and allowed to cool to 60 ^{o}C before media into each petri dish. Wastewater samples were serially diluted in PBS buffer and filtered through 0.45 µm mixed cellulose ether filters (Whatman, Piscataway, NJ) using a vacuum pump. Filters were then placed on the prepared plates were incubated at 35 ºC for 24 h. A single colony with metallic phenotype typical of total coliform was enriched and cultured in Luria-Bertani (LB) broth overnight. The culture was maintained in glycerol at -80 ^{o}C as described above.

Biosand column setup. Bench scale reactors used in the present study were constructed based on the specifications outlined by CAWST (Biosand, 2009). A schematic can be seen in Figure 4.1a and a photo of the reactors is shown in Figure 4.1b. The bioreactors were built using 6” diameter PVC with a total length of 41”. The height from the diffuser plate to the effluent pipe was 30”. Three ports were placed on 2 sides of the filter in order to collect water and sand samples at three different depths from the diffuser plate (i.e., 7”, 14” and 24.5”). The effluent pipe was connected to the bottom of

the filter and was tied to the outside of the pipe at a height 2” above the distribution plate. At this height, the water level inside the reactor rested 2” above the sand layer. The effluent pipe drained to a storage bucket. Each filter was filled with 50 mm (0.912L) of gravel, 50 mm (0.912 L) of coarse sand, and 0.5436 m (9.915 L) of fine sand. All-purpose sand was obtained from a local hardware store. Sand was sieved, washed and dried. Sand captured on the 0.25” sieve was used for the drainage gravel. Sand captured on the 0.04” sieve was used as coarse sand. Sand that passed the 0.3” sieve was used for the sand column.

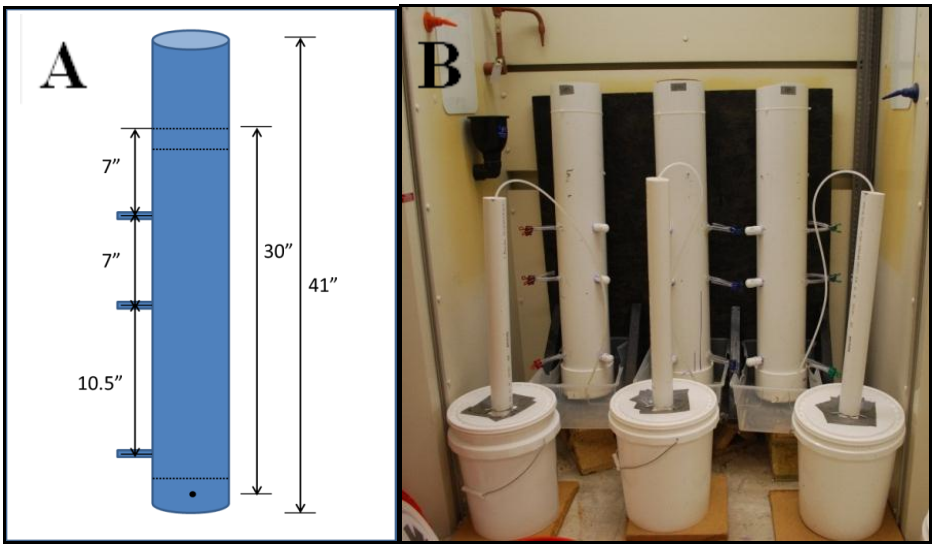


Figure 4.1 A) Schematic of lab biosand filter and B) photograph of laboratory columns.

Sand column operation and sampling. Three columns were operated in parallel. Reactor 1 received lake water with a high TOC concentration (14.2 $\pm$ 0.5 mg/L). Reactor 2 received lake water diluted 1:1 with deionized water to obtain a medium TOC

concentration (7.6 $\pm$ 0.6 mg/L). Reactor 3 received lake water diluted 1:10 with deionized water to obtain a low TOC concentration (2.1 $\pm$ 0.3 mg/L). These conditions were selected to mimic field application of waters with varying TOC concentrations. Different TOC concentrations were used to determine the effect of water source TOC on biosand filter performance. Surface water generally has higher TOC concentrations and deep well ground water can have very low concentrations of TOC (Wetzel, 1975).

Samples were analyzed for TOC concentration via a Shimadzu Total Organic Carbon Analyzer (TOC-L) (Colombia, MD). Concentration and standard deviation was reported. Samples were measured in at least triplicate.

Water was collected once per week from Lake Crabtree (Morrisville, NC). To ripen the biosand filters, single charges consisting of 6 L of water were added to each filter every day, according to the TOC concentration allocation, either high, medium or low concentration of TOC, as described in the previous paragraph. This charge volume was selected as it represented one pore volume or the filter water holding capacity. Six pore volumes were selected as the general guidelines consist of filling up the reactor six times prior to usage as described by others (Clean Water for Haiti, 2011).

Challenge tests were performed periodically (every six charges, based on the guideline described above) to determine the treatment efficacy as a function of charge volume. After every six charges, 6 L of *V. cholerae* cells suspended in PBS buffer were added to each filter consecutively in increasing concentrations: 10^2, 10^3, 10^5 and 10^7 cfu/mL. These concentrations were selected as the infectious dose of cholera is known to be 10^6 cells (Schmid-Hempell, 2007) and these concentrations represent a range of

measured environmental concentrations previously reported in the literature (Kott and Betzer, 1972; AWWA, 2006). Note that concentrations found in Haiti (Chapter 3) ranged from $10^0 – 10^2$ cfu/mL. Each charge was allowed to finish filtering before the next charge was added in order to prevent mixing. Influent and effluent of each pure culture charge was collected. *V. cholerae* concentration was measured by spread plate technique using selective culture medium (i.e., TCBS agar) as described in Chapter 3. This *V. cholerae* challenge test was repeated each time the filter received an additional six charges of lake water or diluted lake water in order to assess the effect of number of charges on filter efficacy. After each *V. cholerae* challenge day, 4" bore samples (0.5" diameter) of the biofilm or schmuztdecke were collected in triplicate and stored at -20 ^{o}C until further processing.

Once per week, influent and effluent were collected to measure TOC, pH, dissolved oxygen (DO), biological oxygen demand (BOD), and heterotrophic bacteria concentration. Flow rate was also measured to assess for clogging. Influent was collected from the water before it was charged to the filter by dipping collection tubes into the bucket in which the water was mixed. Effluent water was collected from the effluent tube immediately after influent water was charged to the filter by placing the collection tubes at the end of the effluent tube. Prior to measurement, TOC water samples were stored at -20 ºC.

DNA isolation. DNA was extracted from schmutzdecke samples using phenol:chloroform DNA extraction. In brief, cells were resuspended in TE buffer,

washed, and resuspended in TE buffer, 10% SDS and Proteinase K. To lyse cells and isolate DNA, cells were vortexed and incubated at 56 °C for 2 h. To purify the DNA, phenol/chloroform extraction was performed twice by adding an equal volume of phenol/chloroform, vortexing gently, centrifuging at 13,000 rpm for 1 min, and removing the aqueous phase and placing in a clean tube. Then, an equal volume of chloroform was added, gently vortexed, centrifuged, and the aqueous phase was placed in a clean tube.

To precipitate the DNA, ethanol precipitation was performed. One M NaCl and ice cold 190 proof ethanol was added, the mixture was mixed by inversion, and incubated in the freezer at -20 ^{o}C overnight. Then, samples were centrifuged at 13,000 rpm for 10 min to form a pellet of DNA. The ethanol mixture was decanted and ice cold 70% ethanol was added and centrifuged to desalt the pellet. The supernatant was removed and once the liquid evaporated, DNA was resuspended in Tris-Cl.

The phenol/chloroform/isoamyl alcohol mixture was obtained from Life Technologies (Carlsbad, CA). Concentration and purity of DNA was measured on a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Community analysis by T-RFLP. PCR was performed, targeting the *16S* gene, with the 6 – carboxyfluorescein-labeled fluorescent forward primer (27F) and reverse primer 1392R. PCR conditions used were 94 ºC for 5 min followed by 35 cycles of 30 s at 94 ºC, 30 s at 50 ºC, and 30 s at 72 ºC, with a dissociation step at the end for quality control. Amplicons were purified utilizing a Qiagen PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Final PCR product

concentrations and purity were measured on a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). One hundred ng of purified PCR product and 10 U of *Msp1* (New England Biolabs, Beverly, MA, USA) were used for each T-RFLP reaction. The mixture was incubated at 37ºC for 2 h. Analysis of fragments was performed using an Applied Biosystems 3100 capillary sequencer (Foster City, CA) with POP6 polymer and ROX-labeled MapMarker 1000 size standards (Bioventures, Inc., Murfreesboro, TN) at the Duke University DNA Analysis Facility (Durham, NC). Standard procedures were followed.

In order to perform principal component analysis (PCA) and non-metric multidimensional scaling (nm-MDS), T-RFLP profiles were visualized using Applied Biosystems GeneScan v3.7.1 software (Foster City, CA). T-REX online (Culman et al., 2009) software was used to process raw data through T-RF alignment to look at presence/absence of fragments. Any fragment 50 bp or smaller was excluded from data set to ensure no primer dimers were included in the analysis. Ordination plots were analyzed for group clustering using the Paleontological Statistics software (PAST) statistical software to determine if ordination clusters were statistically similar (Hammer, Ø. & Harper, D.A.T. 2006. Paleontological Data Analysis. Blackwell.).

EPS extraction and analysis. EPS was extracted using a cation exchange resin as previously described (Frolund et al, 1996; Badireddy et al, 2011). Briefly, 75 mg of the Na^+ form of a polystyrene divinylbenzene microporous ion exchange resin (Dowex 50WX8, 20e50 mesh, Sigma Aldrich)/g volatile suspended solids (VSS) was added to 50

mL of sample and shaken at 900 rpm for 4 h at 4 ^{o}C. A two-step centrifugation at 4 ^{o}C (first at 5000g for 15 min and then at 12,000g for 30 min) followed by filtration using a 0.45 mm cellulose acetate membrane was used to remove resin, microorganisms, and residual debris to obtain an EPS sample for further analysis. After collection from each biosand filter, samples were stored at -20 ^{o}C until further analysis.

Protein was quantified by the Modified Lowry Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin standards. Briefly, 40 µL of standard or sample was plated on a microplate (Thermo Fischer Scientific) in duplicate. Two hundred µL of Modified Lowry Reagent was added to each well, and the plate was shaken at 1500 rpm for 30 s using a Thermo Electron Corporation Multiskan MCC (Waltham, MA) plate reader. Then 20 µL of prepated 1X Folin-Ciocalteu reagent was added, and the plate was shaken at 1500 rpm for 30 s. The plate was covered and incubated at room temperature for 30 min. Then the absorbance was recorded at 690 nm.

Carbohydrates were measured using the phenol-sulfuric acid method against glucose standards (Bariddy et al, 2011). Briefly, 10 µL of standard or sample was plated on a microplate in duplicate. Five µL of 80% phenol solution (w/v) was added to each well and the plate was shaken at 1500 rpm to mix. Then 200 µL of sulfuric acid was added to each well in a stream. The plate was incubated at room temperature for 10 min and the absorbance was recorded at 490 nm.

Uronic acid was determined as described in Bariddy et al (2011). Briefly, 40 µL of standard or sample was added to a microplate in duplicate. Two hundred µL of sulfuric acid (96% w/w) containing 120 mM sodium tetraborate was added. The plate was shaken

at 1500 rpm to mix and then incubated at 80 ^{o}C for 1 h. Then 100 µL of m-hydroxydiphenol was added to each well, the plate was shaken at 1500 rpm, and incubated at room temperature for 15 min. The absorbance was measured at 540 µm.

Sorption analysis. This analysis was performed in order to isolate the effect of physical/chemical attachment from schmutzdecke-based filtration. At the end of the experimental phase, the 3 sand column packing materials were compared to determine how TOC loading impacts physical/chemical attachment. New sand (not previously exposed to waters with TOC) was used as a control to determine the effect of physical/chemical attachment during early biosand filter operation prior to schmutzdecke development. After the biosand filters were shut down (66 ripening charges with TOC; 11 *V. cholerae* challenges), grab samples of packing media throughout the depth of each filter (excluding the first four inches which presumably housed the schmutzdecke) were collected and stored at 4 ^{o}C until further processing.

Tests were performed in PVC pipes (2.5 cm diameter, 76 cm long) packed with 432 g of each filter's sand (representing the total mass of the grab sample that was stored). A fourth pipe was packed with newly sieved and washed sand. *V. cholerae* pure cultures suspended in 1 L PBS buffer at a concentration of $5.60 \times 10^{6} \pm 4.05 \times 10^{5}$ cfu/mL was added to each column and removal efficiency was measured. This concentration was used as it represents the higher end of *V. cholerae* concentration that would be found in environmental samples. Effluent was collected in 140 mL increments. This volume was selected as it represents 1/7 of the total volume added to each column.

Influent and effluent samples were serially diluted in PBS buffer, and plated on TCBS agar using spread plate technique as described in Chapter 3. Plates were incubated at 37 ^{o}C for 24 h.

Statistical Analysis. The unpaired, two tailed student's t test was used to identify statistical differences between samples. Results were considered statistically different when the p-value <0.05.

4.3 Results and Discussion

TOC and number of charges affect removal efficacy of V. cholerae in lab biosand filters. Filters receiving influent waters with different TOC concentrations exhibited different removal efficiencies (Figure 4.2). In general, removal efficiency increased as TOC concentration decreased. Overall, for influent *V. cholerae* charges of 10^2, 10^3, 10^5 and 10^7 cfu/mL, the filter receiving high TOC influent had statistically higher effluent concentrations (p>0.05) of *V. cholerae* than the filters receiving medium and low TOC influents. For 10^2, 10^3, 10^5, and 10^7, removal efficiency was consistently highest in the filter receiving low TOC and lowest in the high TOC treatment. For the highest *V. cholerae* loading, the average effluent concentration was $9.89 \times 10^6 \pm 7.48 \times 10^6$ cfu/mL for the high TOC loading and $2.60 \times 10^6 \pm 3.37 \times 10^6$ cfu/mL for the low TOC loading. This is a 73.7% difference ($p = 9.49 \times 10^{-3}$), suggesting that TOC in influent waters is a critical parameter controlling removal efficiency. Additionally, for all *V. cholerae*

charges, the filter receiving medium TOC had statistically higher effluent concentrations than the filter receiving low TOC ($p<0.05$).

At low *V. cholerae* concentrations, large breakthrough events occurred occasionally in filters receiving medium and high TOC loadings (Figure 4.2). These breakthrough events are problematic, because they imply that the filter could in fact deliver an infectious dose when not using one would not. However, the filter receiving low TOC never experienced any breakthrough throughout the course of the study. We hypothesized that this occurred because there are other major removal mechanisms other than the schmutzdecke at work in the low TOC filter, such as physical/chemical attachment, that contribute to *V. cholerae* removal efficiency. Alternatively, these data may suggest that the same removal mechanisms are at work, regardless of TOC loading, but TOC is the main thing removed in the filter receiving high TOC, rather than *V. cholerae*. This hypothesis is further explored below.

It should be noted that effluent samples were obtained by collecting the first 100 mL of effluent. If the full 7L effluent volume was collected and the average effluent plated, or if effluent samples were plated every 1L of effluent, more breakthrough events may have been observed.

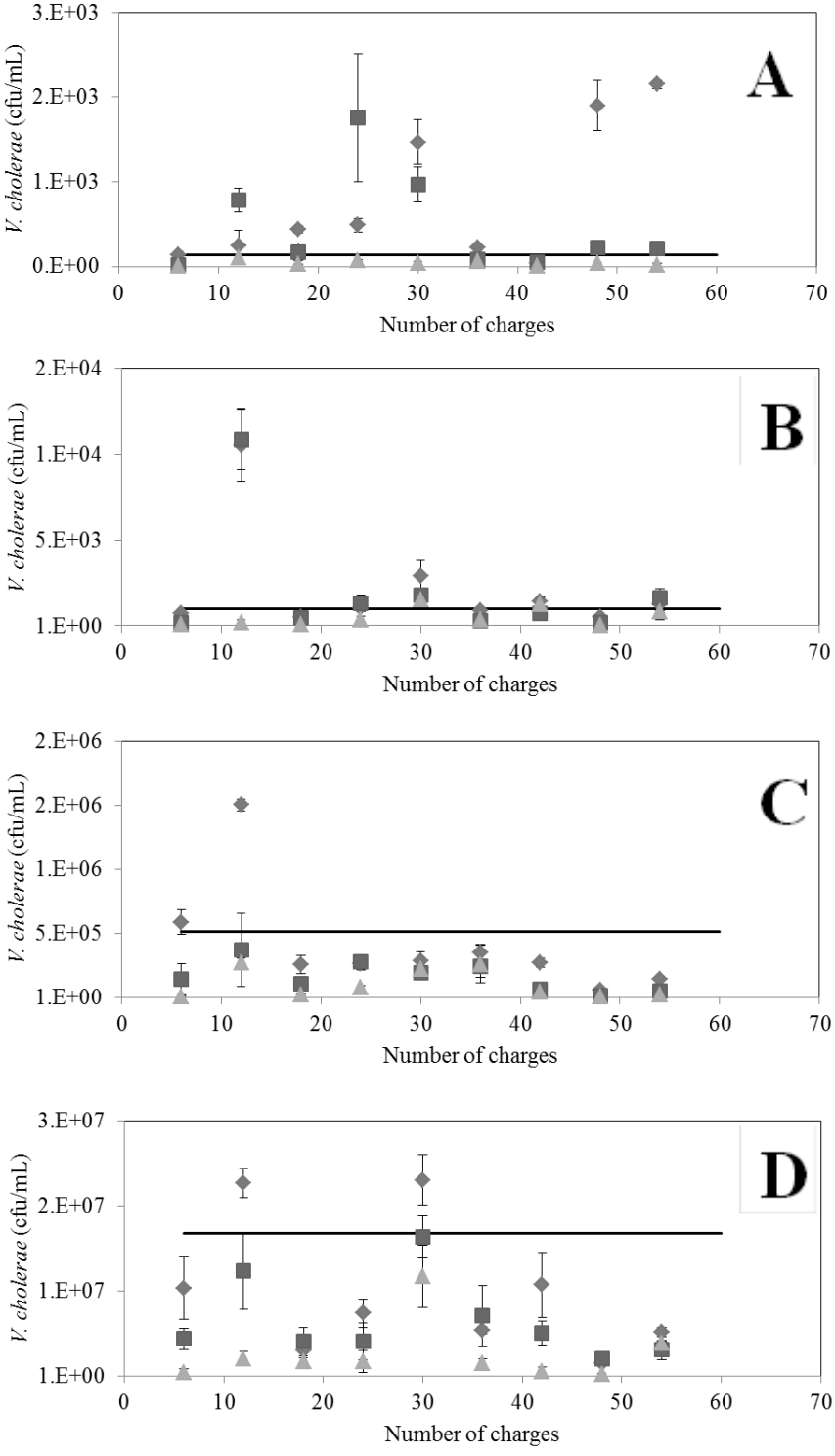

A
B
C
D
V. cholerae (cfu/mL)
Number of charges
3.E+03
2.E+03
1.E+03
0.E+00
2.E+04
1.E+04
5.E+03
1.E+00
2.E+06
2.E+06
1.E+06
5.E+05
1.E+00
3.E+07
2.E+07
1.E+07
1.E+00
0
10
20
30
40
50
60
70

Figure 4.2 Influent (▬)and effluent concentrations from high TOC (◆), medium TOC (■) and low TOC (▲) for pure culture *V. cholerae* charge influent concentrations of A) 10^2 cfu/mL, B) 10^3 cfu/mL, C) 10^5 cfu/mL, and D) 10^7 cfu/mL. Error bars represent the standard deviation of triplicate samples.

Treatment performance in all reactors initially drops down and then rebounds (Figure 4.3). As described below, the initial decrease is attributed to physical removal processes whereas the rebound is linked to biological treatment. At high concentrations of *V. cholerae* in the influent (10^7 cfu/mL), the number of charges also affected removal efficiency (Figure 4.3). For medium and low concentrations of TOC, removal efficiency gradually decreased up to charge 30 then generally increased until the end of the study. Early removal patterns (prior to day 30) may be due to the effect of physical/chemical attachment. The sand within the filters receiving medium and low TOC waters had more sorption sites available compared to the sand within the filter receiving high TOC, since organic carbon will sorb to sand particles and use up sorption sites as demonstrated by others (Jardine et al, 1989; Moore et al, 1992). As filter operation time progressed up to charge 30, less sorption sites also were available within the lower TOC filters as organic carbon may have been taking up those sorption sites leading to a reduced treatment efficacy. After charge 30, removal efficiency in all three filters increased due to the increasing effect of the schmutzdecke (biofilm) (Figure 4.4). By the end of the study, each filter was achieving 95% removal or greater of *V. cholerae*, indicating that TOC concentration may not be a critical parameter controlling removal after steady state is reached.

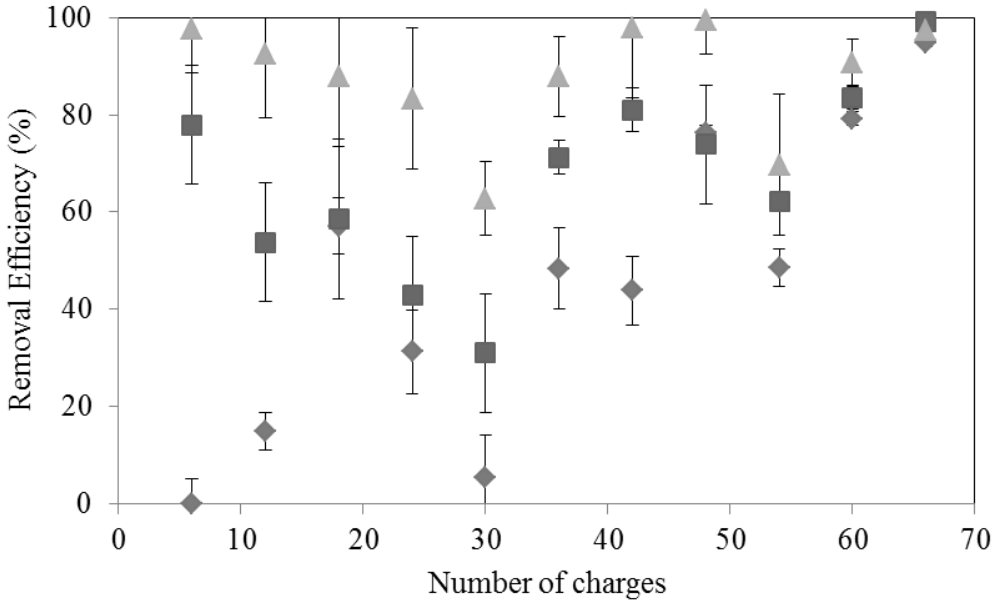


Figure 4.3 Removal efficiency during 10^7 cfu/mL *V. cholerae* challenge for high TOC (◆), medium TOC (■) and low TOC (▲). Error bars represent the standard deviation of triplicate samples.

As shown in Figure 4.4., biofilms in filters receiving high and medium TOC waters had a gradual decrease in EPS up to charge 30 (starting concentrations of 0.546 and 0.966 mg of EPS per g VSS, respectively) and then experienced a gradual increase over time (up to 0.922 and 0.450 mg of EPS per g VSS, respectively). The filter receiving low TOC water had an increase in EPS up to charge 24 (beginning at 0 mg of EPS and increasing to 0.474 mg of EPS per g VSS), then a decrease at charge 30 with a gradual increase with time after that, then a decrease near the end of the study (up to 0.505 mg EPS at charge 48 and then down to 0.184 mg EPS per g of VSS at charge 66). The trend suggests that as EPS content increases, *V. cholerae* removal improved and provides additional support that the treatment in the latter part of the study had a large biological

component. These data indicate the importance of the schmutzdecke layer in biosand filter performance. The EPS quantity levels out in all filters around charge 42, with averages of 1.48 $\pm$ 0.33, 0.71 $\pm$ 0.35, and 0.56 $\pm$ 0.25 mg EPS per g VSS for the high, medium and low TOC filters, respectively. From charge 42 to charge 66, the high TOC filter had significantly more EPS than the medium ($p = 0.003$) and low ($p = 0.022$) filters, but the difference in amount of EPS between the medium and low filters was not statistically significant ($p>0.05$). The filter receiving low TOC water consistently had the lowest concentration of EPS, whereas the filter receiving high TOC water had the highest amount of EPS near the end of the study. This trend was expected as TOC provides nutrients for a growing biofilm (Chandy et al, 2001; Delille et al, 2007). However, as discussed above, the low TOC filter consistently had greater removal efficiencies than the high TOC filter, suggesting that physical/chemical attachment may be a larger contributor to treatment efficacy in some instances.

The filter receiving medium TOC water did not exhibit a clear EPS quantity trend over time. This may be due to biofilm shearing during operation, which is known to occur (Speitel et al, 1987). This shearing may have happened specifically in the medium TOC and not the high or low TOC receiving filters as the medium TOC had more biofilm that the low TOC and a higher pore volume velocity than the high TOC (indicated by the higher flow rate), making it more prone to shearing.

After charge 66 and the 11th *V. cholerae* challenge, a total coliform challenge was conducted involving four consecutive influent charges of total coliform suspended in PBS buffer at concentrations of 10^2, 10^3, 10^5 and 10^7 cfu/mL. Up until charge 66, the

schmutzdecke in each filter was only exposed to lake water or diluted lake water microbial communities and pure cultures of *V. cholerae.* The total coliform challenge involved four consecutive charges of pure culture total coliform over 15 h, giving the total coliform time to compete with the established biofilm community. EPS quantity drastically dropped after these charges in the high and medium TOC biosand filters (from 0.922 to 0.545, and from 0.450 to 0.097 mg of EPS per g of VSS for high and medium TOC, respectively), suggesting that the biofilm may be sensitive to abrupt changes in microbial community input due to interspecies competition. Others have previously reported similar findings (Rau et al, 2005; Kreth et al, 2005). It is important to note that microbial communities in water sources can drastically change from wet to dry season (Wright, 1986), and thus filter performance may vary as seasons change. It is common practice for biosand filter users to change water sources from wet to dry seasons (Singh et al, 2001; Tiwari et al, 2009), and this may warrant additional studies to determine this effect. Interestingly, no drop was observed in the low TOC biosand filter after the total coliform challenge (from 0.184 to 0.194 mg EPS per g of VSS). This finding may be due to a more robust community establishment in the present study and further studies should be performed to confirm whether or not this finding is linked to TOC loading or simply an artifact of our study.

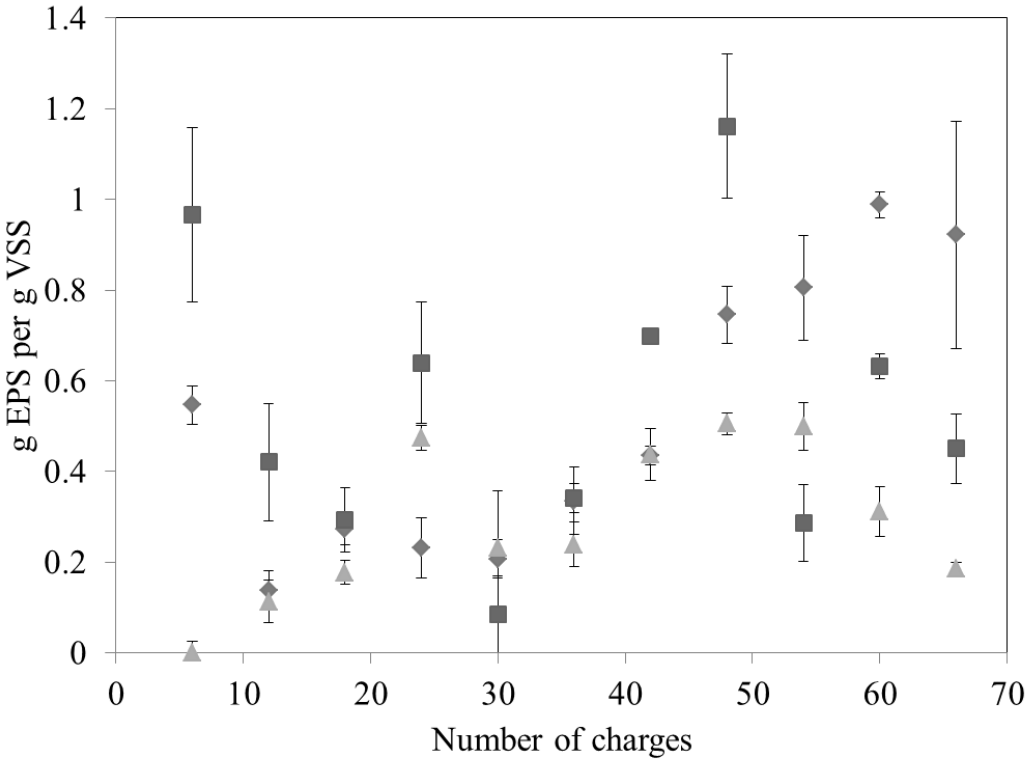
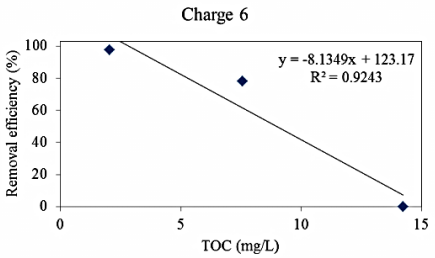
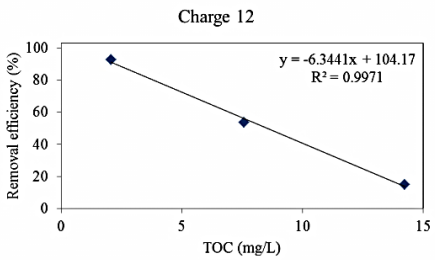


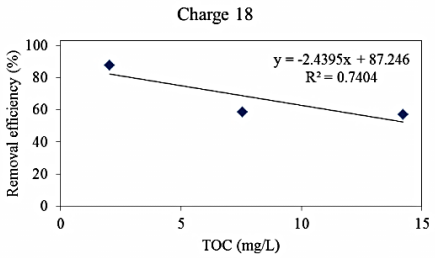
Figure 4.4 Gram of EPS per gram of VSS versus number of charges for high (◆), medium (■), and low (▲) TOC. Error bars represent the standard deviation of duplicate samples.

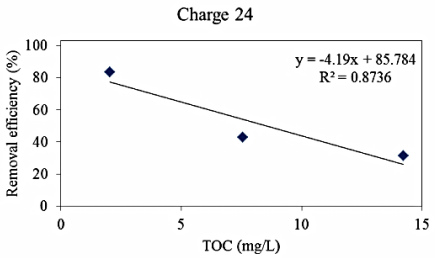
These data suggest that TOC concentration may play a role in biosand filter performance. To further support these findings, a correlation analysis was carried out between TOC and *V. cholerae* removal efficiency at the time each challenge test was performed (Figure 4.5). A strong linear relationship between TOC and *V. cholerae* removal efficiency was observed at the highest *V. cholerae* loading (10^7 cfu/mL). Over time, however, TOC amount becomes less significant with time suggesting that this criteria is especially important for predicting treatment efficacy following startup of

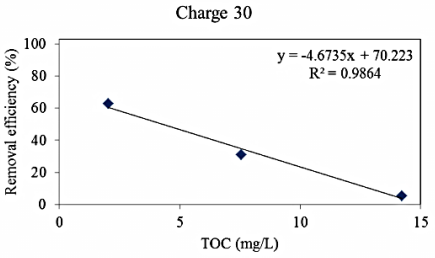
biosand filters. Similar findings were observed for the other *V. cholerae* loadings (Figure 4.6).

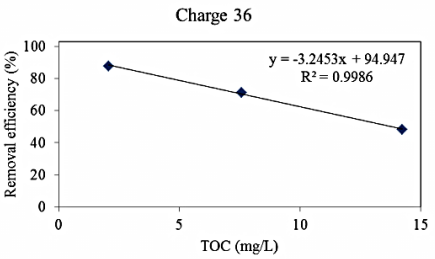

Charge 6
y = -8.1349x + 123.17
R² = 0.9243
Removal efficiency (%)
TOC (mg/L)

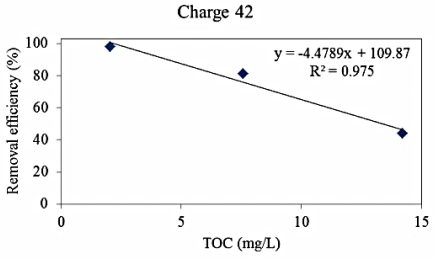

Charge 12
y = -6.3441x + 104.17
R² = 0.9971
Removal efficiency (%)
TOC (mg/L)

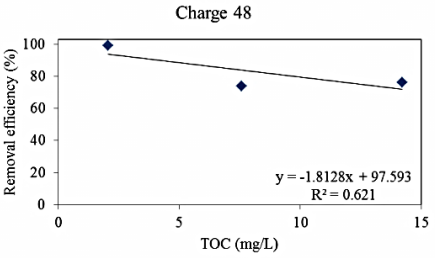

Charge 18
y = -2.4395x + 87.246
R² = 0.7404
Removal efficiency (%)
TOC (mg/L)

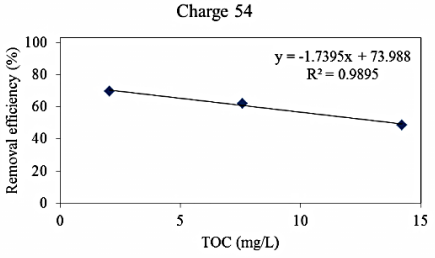

Charge 24
y = -4.19x + 85.784
R² = 0.8736
Removal efficiency (%)
TOC (mg/L)

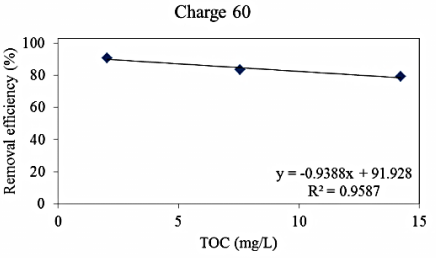

Charge 30
y = -4.6735x + 70.223
R² = 0.9864
Removal efficiency (%)
TOC (mg/L)


Charge 36
y = -3.2453x + 94.947
R² = 0.9986
Removal efficiency (%)
TOC (mg/L)


Charge 42
y = -4.4789x + 109.87
R² = 0.975
Removal efficiency (%)
TOC (mg/L)


Charge 48
y = -1.8128x + 97.593
R² = 0.621
Removal efficiency (%)
TOC (mg/L)


Charge 54
y = -1.7395x + 73.988
R² = 0.9895
Removal efficiency (%)
TOC (mg/L)


Charge 60
y = -0.9388x + 91.928
R² = 0.9587
Removal efficiency (%)
TOC (mg/L)

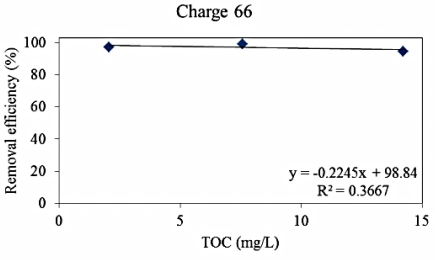

Charge 66
y = -0.2245x + 98.84
R² = 0.3667
Removal efficiency (%)
TOC (mg/L)

Figure 4.5 Removal efficiency of *V. cholerae* versus TOC over time in operation. *V. cholerae* loading of 10^7 cfu/mL.

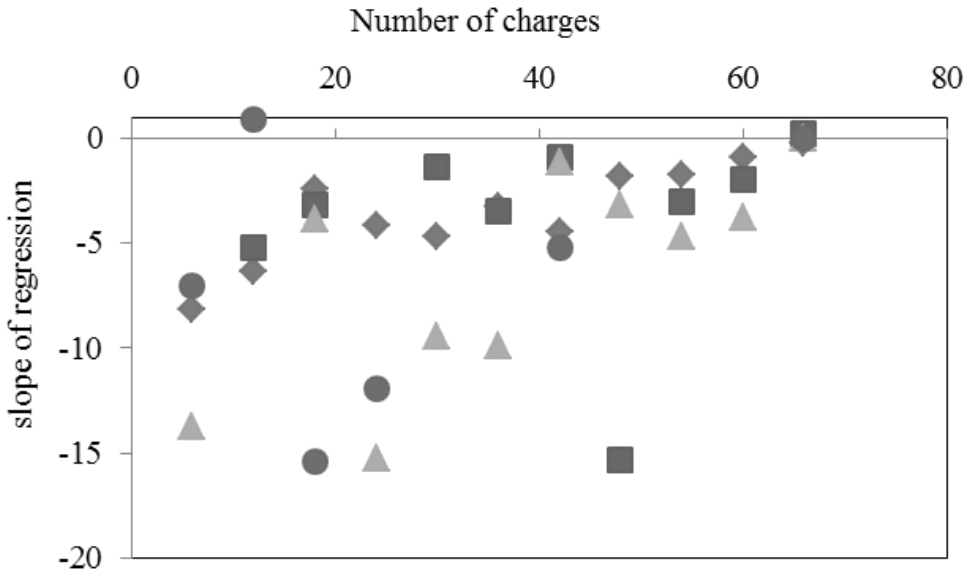


Figure 4.6 Slope of the linear regression between TOC and *V. cholerae* removal efficiency versus number of charges for each *V. cholerae* loading, 10^2 cfu/mL (●), 10^3 cfu/mL (▲), 10^5 cfu/mL (■), and 10^7 cfu/mL (◆).

It should be noted that at the end of the study, although each filter was achieving high percentage removal rates, the effluent concentration for high, medium and low TOC, respectively were still approximately 10^5 cfu/mL, which is above the infectious dose based on a consumption of 2 L per day (Schmid-Hempell, 2007). Thus, biosand filter performance should not be based on percent removal, but effluent concentrations in order to accurately reflect the technology's capacity to provide safe water for users.

Microbial community structure of the schmutzdecke. Microbial community structure clustered based on TOC concentration (Figure 4.7). The community receiving high TOC showed the tightest cluster, indicating a more similar community over time in operation as compared to the lower TOC receiving communities. The schmutzdecke receiving the lowest TOC waters exhibits the most vertical spread, suggesting more diversity in the community over time in operation. Analyzing the nm-MDS plot shown in Figure 4.6 with PAST shows that TOC concentrations explains 90.5% of the microbial community differences while the amount of EPS only explains 9.2% of the microbial community differences. Using the coordinates generated by PAST for the nm-MDS plot, we can graph each microbial community coordinate with the TOC concentration and we see that there is a strong linear relationship ($R^2 = 0.9723$) (Figure 4.7). This suggests that TOC concentration plays a large role in microbial community structure of the schmutzdecke. This was not observed in the field (Chapter 3) likely because the source waters were so different so a much larger field study would be needed to reach these same conclusions.

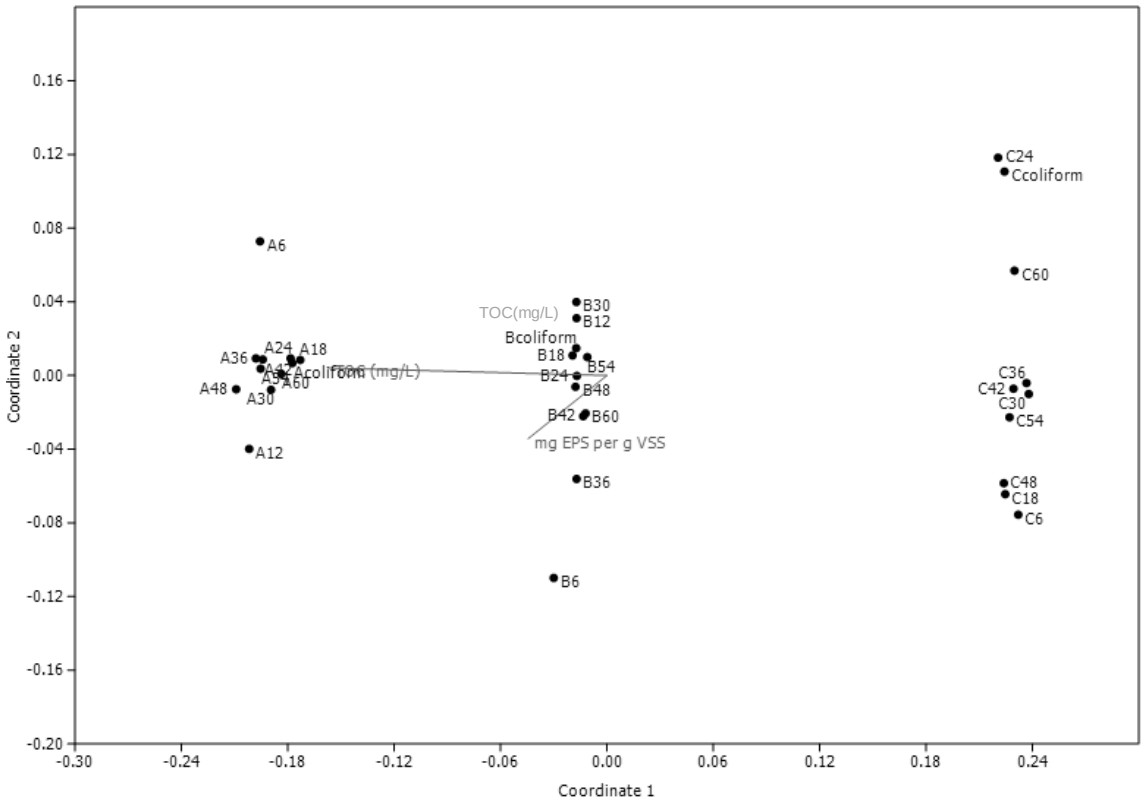


Figure 4.7 Non-metric multi-dimensional scaling (nm-MDS) of biosand filter schmutzdecke microbial community with two environmental factors: TOC concentration (mg/L) and amount of EPS (mg EPS per g VSS).

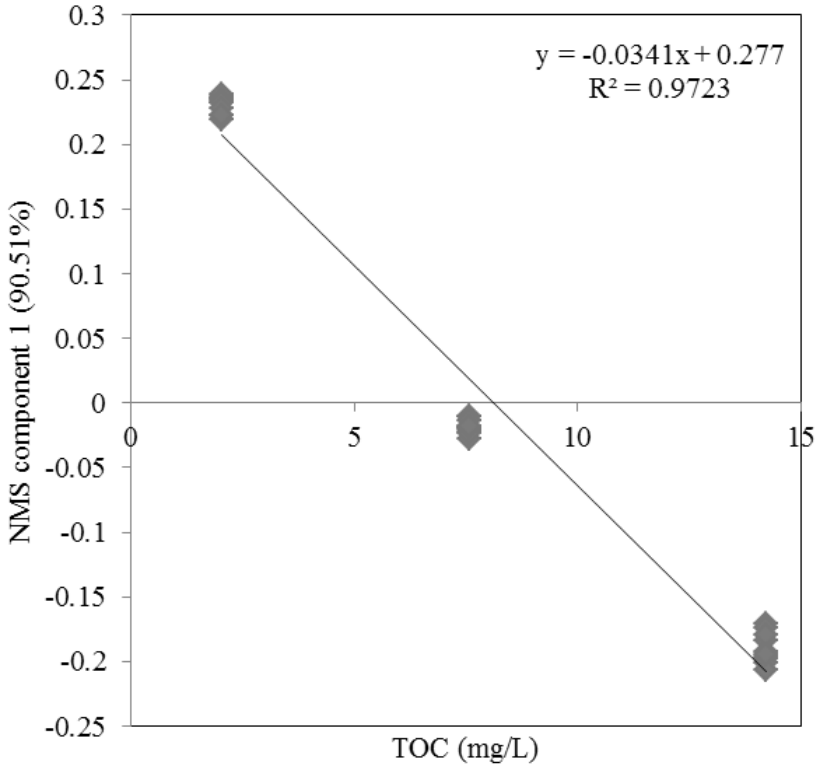


Figure 4.8 NMS component 1 versus TOC concentration.

Physical/chemical attachment plays an important role in removal efficiency during biosand filter start up. Sand samples from the three filters facilitated low levels of attachment compared to new sand (Figure 4.8). This was expected as total available sorption sites will be reduced as more TOC and bacteria are added to a specific volume of packing material. The new sand consistently achieved a C_{out}/C_{in} value below 0.1, indicating high removal efficiencies (>90%) in new sand due to physico-chemical sorption.

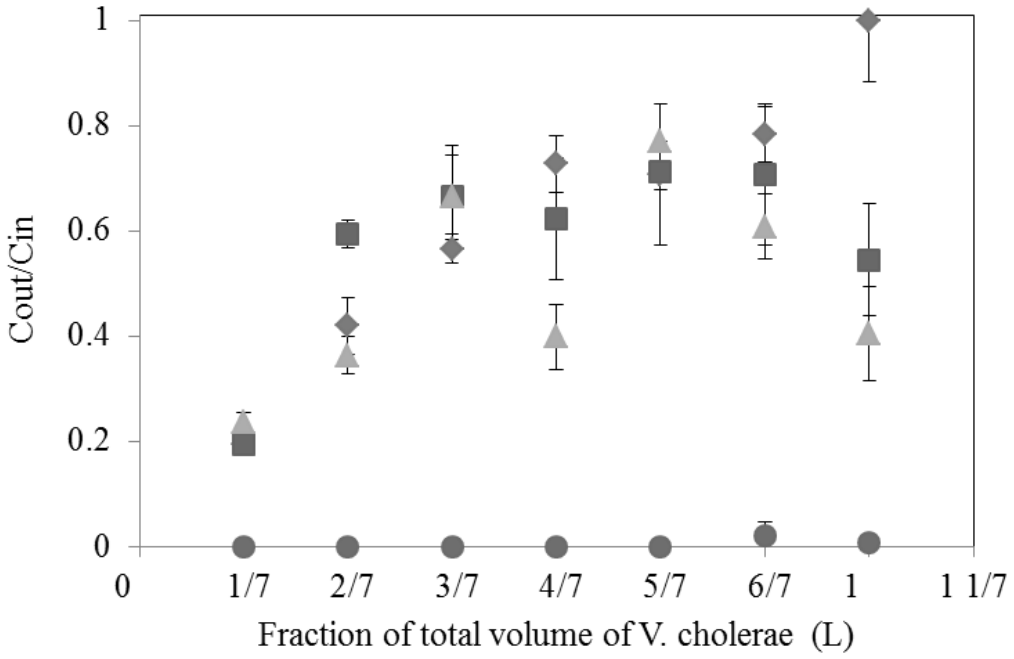


Figure 4.9 Sorption capacity of sand in biosand filters receiving high (◆), medium (■), and low (▲) TOC. (●) indicates newly washed and sieved sand. Error bars represent the standard deviation of duplicate samples.

For comparison purposes, sorption rates were calculated assuming that biosand filter columns can be modeled as plug flow reactors, described by the following equation:

$$\frac{Cout}{Cin} = e^{-k\tau}$$

where C_{out} is the concentration of the effluent, C_{in} is the concentration of the influent, τ is the residence time, and k is the rate of sorption. As expected, the newly sieved and washed sand has the highest attachment rate of 0.1062 $\pm$ 0.009 min^{-1}, which is at least three times greater than attachment rates for the used packing media. The high,

medium, and low TOC receiving sands have attachment rates of 0.0212 $\pm$ 0.002, 0.0212 $\pm$ 0.002, and 0.0357 $\pm$ 0.003 min^{-1}, respectively, indicating that of the three filters, the one receiving the lowest TOC was able to accomplish the highest physical/chemical attachment of *V. cholerae* confirming our previous findings. The medium and high TOC receiving sands had the lowest sorption rates, which explains the bacterial breakthrough events from the *V. cholerae* challenge tests previously discussed. The sand receiving the lowest TOC influent had the highest physical/chemical attachment rate of the three biosand filter sands, which is consistent with the lack of bacterial breakthrough events observed in the *V. cholerae* challenge tests.

4.4 Conclusions

In this chapter, we have identified important parameters controlling removal in the biosand filter, including: time in operation, TOC loading, amount of EPS, and physical/chemical attachment. Under low TOC, physical/chemical attachment may be more important than schmutzdecke effects, indicated by the low amounts of EPS, high physical/chemical attachment reaction rates, and high removal efficiencies of *V. cholerae*. Under high TOC, schmutzdecke effect, particularly EPS amount, is more important for removal than physical/chemical attachment. This is significant, as according to these data, source water characteristics will determine mechanisms of removal as well as removal efficiency of *V. cholerae* within the biosand filter. These data point to one of the challenges of point-of-use treatment, behavioral factors. User behavior can affect biosand filter performance and confound treatment efficacy data,

including amount of water passed through the filter, idle time, how often the filter is cleaned (schmutzdecke disturbance), and, here we show, the use of multiple water sources.

Chapter 5. Cocopeat as a packing media for wastewater treatment in the developing world: optimizing redox depths for nitrogen and phosphorus removal in vertical flow biofiltration columns

5.1 Introduction

Increased attention and effort by the global community has led to an increase in access to water treatment technologies over the last 22 years. The Joint Monitoring Program by the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) have declared that the world met the Millennium Development Goal (MDG) target for drinking water. However, it is projected that the MDG target for sanitation will likely be missed (WHO/UNICEF, 2014). Over 1.8 billion people have gained access to improved sanitation since 1990, but there are still 2.5 billion people without access (WHO/UNICEF, 2014). On-site sanitation technologies can provide an interim solution. One such method is biofiltration. Naturally occurring media, such as peat, can be utilized in fixed film systems, where the attached growth, colonization and reproduction of microorganisms are promoted for the treatment of wastewater streams (Sherman, 2006).

Peat filtration has been used for municipal wastewater treatment for many decades (Gutenspergen et al 1980, Farnham and Brown 1972, Nichols and Boelter 1982), as well as on-site treatment of household wastewater from septic tanks and pit latrines (Nichols et al 1982, Rock et al 1984, Brooks et al 1984). Peat-packed biofilters have shown to be effective at removing nutrients, biological oxygen demand and coliform bacteria from wastewater streams (Corley 2006, Viraraghavan 1987, Kennedy 1999,

Kennedy 2000). Naturally occurring media such as peat offer a benefit over artificial media such as foam, textiles and plastic because they utilize naturally occurring flora and fauna. However, often these natural media must be mined, which can have critical negative impacts on the environment, sustainability and local ecosystems.

Cocopeat, a readily available product in Southeast Asia, is a by-product of coconut processing plants and comes from the outer husk of the coconut (Verdonck 1983). The coconut shell is shredded and then the fibers are removed. It contains approximately 30% fibers and the remaining 70% is ground pith, which has a soil-like texture. Cocopeat has an advantage over sphagnum peat, as it does not need to be mined, while seemingly maintaining physical properties similar to sphagnum peat, possibly making cocopeat a suitable packing medium for biofiltration.

While cocopeat may be an ecologically sustainable and locally available packing medium suitable for biofiltration, it is necessary to evaluate how it compares to other traditional packing media. In this study, cocopeat was compared to sphagnum peat and Celite® in lab scale biofilters treating simulated septic tank effluent. Microbial fingerprints were obtained and compared between the different packing media to determine if similar community profiles and robustness were established. Also, the effect of promoting different redox zones in the biofilters was evaluated with the goal of improving nitrogen and phosphorus removal efficiency.

5.2 Material and Methods

Bioreactor Design. Six lab scale column reactors were built based on previously published research (Patterson, 1999). Solid white plastic PVC pipe with a 4" diameter and 29.5" height was used for the main body of the bioreactors. The height from the top of the reactor to the effluent pipe was 29". Three sampling ports were placed on two sides of the length in order to obtain water and peat samples at different depths. As shown in Figure 5.1a, the ports were placed at 9.5", 16" and 24.5" from the top. Influent wastewater was sprayed intermittently over the media by a ½" cap drilled with small holes via a timer (Titan controls, Vancouver, WA) and a Masterflex pump (Cole-Parmer, Vernon Hills, IL). The effluent drained out of the bottom through a 3/16" inner diameter clear vinyl tube placed at the bottom of the reactor. Each biofilter was packed with a 10 cm deep base layer of 0.187" – 0.5" gravel in order to allow water to pool at the bottom of the reactor for easier drainage. The gravel used in this study was obtained from a hardware store and sieved. On top of the gravel layer, each packing medium (i.e., cocopeat, sphagnum peat or Celite®) was placed to a height of 24". Each bioreactor was prepared in duplicate. A photograph of the experimental setup is shown in Figure 5.1b.

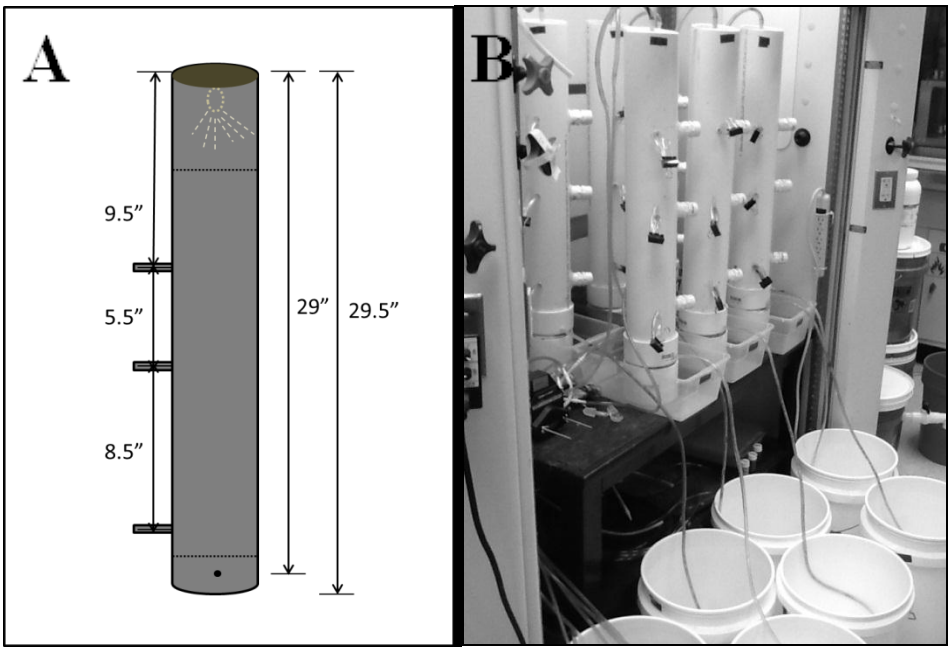


Figure 5.1 A) Schematic of peat reactor and B) photograph of experimental setup

Jar Tests. Baseline sorption and desorption rates for nitrogen and phosphorus were compared for the three packing media. Each test was performed by filling Erlenmeyer flasks with 100 mL of deionized water. One g of each packing medium was added to individual flasks, in duplicate. Flasks were shaken at 60 rpm for 2 h. Liquid samples were then collected and filtered through 0.45 µm mixed cellulose ether filters (Whatman, Piscataway, NJ) for analysis (Viraraghavan, 1987). Ammonium and phosphate concentrations were measured and compared before and after addition of the packing media using methods described below.

Bioreactor Operation. Approximately 4.14 L of activated sludge inoculum obtained from the North Durham Wastewater Treatment plant (Durham, NC) was used to

seed the bioreactors with an active microbial community. During inoculation, the wastewater was added at a flow rate of 6 mL/min over a period of 11.5 h. Following inoculation, the reactors were charged with simulated septic waste at a rate of 1.4 mL/s for 10 s every 2 h, based on previous work (Corley, 2006). A modified simulated septic wastewater medium was used in this study and was based on a recipe developed by Zeng et al (2003). Two L of the simulated wastewater consisted of 100 mL of solution A and 1.9 L of solution B. Solution A contained 17.53 g/L NaAc,1 g/L MgSO4, 0.45 g/L CaCl2, 3.3 g/L NH4Cl, 0.5 g/L Peptone and 6 mL/L of a nutrient solution (recipe provided below). Solution A was adjusted to pH 5.5 with 2 M HCl and autoclaved. Solution B contained 28.5 mg/L KH2PO4 and 32.5 mg/L K2HPO4 and was adjusted to pH 10 with 2 M NaOH. The nutrient solution consisted of 1.5 g/L $FeCl_3$, 0.15 g/L H_3BO_3, 0.03 g/L $CuSO_4$, 0.18 g/L KI, 0.12 g/L $MnCl_2$, 0.06 g/L Na_2MoO4, 0.12 g/L, $ZnSO_4$, 0.15 g/L $CoCl_2$, and 10 g/L ethylenediamine tetraacetic acid (EDTA). All chemicals were obtained from Sigma Aldrich. The simulated wastewater was prepared daily and added to the column using a metered pump (Masterflex, Cole-Parmer, Vernon Hills, IL).

To optimize nutrient removal, various hydraulic residence times were tested to modify redox conditions along the column depth and promote the development of aerobic, anoxic and anaerobic zones. Hydraulic residence times were modified by moving the effluent tube to different heights thereby increasing the amount of time wastewater remained in the biofilters. As shown in Figure 5.2, four different water level depths were analyzed: 0, 5.5, 14 and 19.5” corresponding to the four phases of the

experiment. Phase I consisted of a fully aerobic treatment. Phase II consisted of mostly aerobic treatment with a short anoxic treatment. Phase III consisted of aerobic and a longer anoxic zone while Phase IV consisted of aerobic, anoxic and anaerobic treatment. The hydraulic residence times for the various phases were 0.1, 1.1, 2.8, and 4.0 d for Phases I, II, III and IV, respectively. After steady state was reached for each Phase, 15 mL of packing media samples were collected at each sampling port as well as the top of the bioreactor to analyze for microbial community shifts. In addition, total suspended solids (TSS), volatile suspended solids (VSS), biological oxygen demand (BOD), dissolved oxygen (DO), pH, nitrogen (ammonium, nitrate, and nitrite) and phosphorus (phosphate) concentrations were measured in the influent and effluent wastewater as described below.

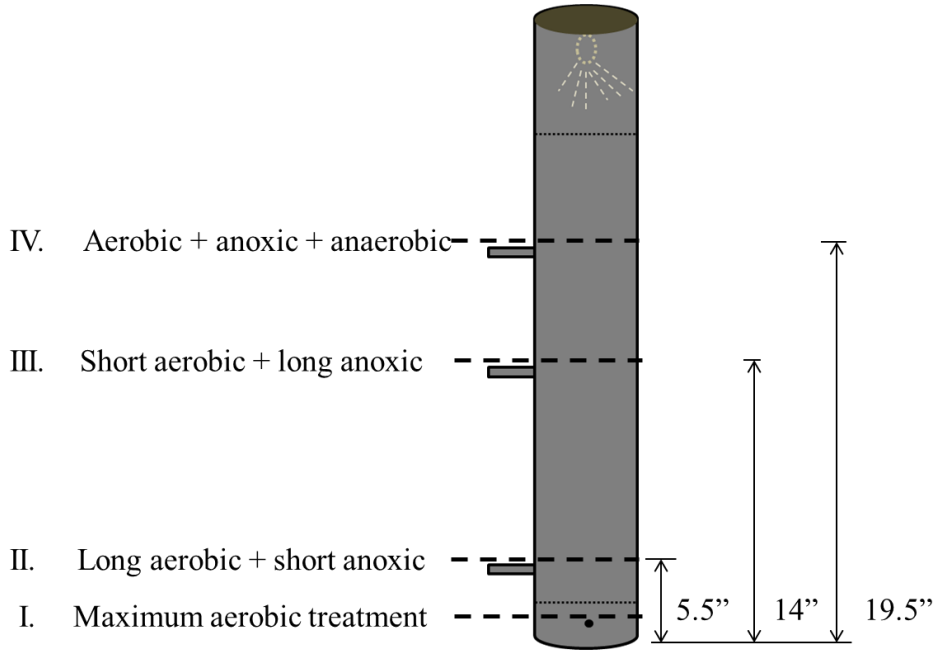


Figure 5.2 Water level at each of the four phases

Analytical Measurements. TSS and VSS were measured according to standard methods as described in IWA, 1999). Briefly, 0.2 µm glass microfiber filters (VWR, Radnor, PA) were prepared by rinsing with 20 mL of deionized water three times. Then, filters were incubated at 105 ºC and 550 ºC to remove any dust or other particles. Influent and effluent were analyzed. Filters were first weighed to determine initial mass. Then, 500 mL of each wastewater sample was filtered using a vacuum pump. The filters were incubated at 105 º C for 1 h and weighed to determine the mass of TSS. Next, filters were incubated at 550 º C for 1 h and then weighed to determine VSS. TSS and VSS for the influent synthetic wastewater and the effluents from each bioreactor were measured in duplicate.

BOD was measured in 300 mL glass BOD bottles using standard methods (EPA, Method 5210 B). Briefly, approximately 0.16 g of Hach nitrification inhibitor was added to each 300 mL bottle (Loveland, CO). Hach BOD nutrient buffer pillows were added to each bottle (Loveland, CO). Bottles were filled to the neck and shaken to mix in the nitrification powder and the nutrient buffer. Then, the initial DO was recorded in duplicate. Deionized water was added to fill the bottle and then the bottles were sealed tightly with glass caps. Bottles were incubated at room temperature (~ 20 ºC) for 5 d. Bottles were then uncapped and the DO was recorded immediately in duplicate using an Oakton DO 6 Acorn series meter (Thermo Fisher Scientific). Measurements were taken in duplicate. Dissolved oxygen measurements were obtained for each columns effluent using the same 300 mL glass bottles. To this end, effluent from the columns was collected in a 300 mL BOD bottle and immediately tested for dissolved oxygen.

Ammonium, nitrite, nitrate and phosphate were all measured on 96 well plates using colorimetric assays as previously described in the literature (Holzem, 2014; Tu, 2010; Hernandez, 2003). Concentrations of each nutrient were calculated in both influent and effluent samples by comparing the optical density of each test to the optical density of known standards. For ammonium, nitrate, nitrite and phosphate, the standards were ammonium sulfate, potassium nitrate, sodium nitrite, and potassium phosphate, respectively. These standards were serially diluted, to allow for a range of optical densities correlating to concentrations to be generated (Figures B1 – B4).

Ammonium concentration was measured by following a modified procedure from standard methods (Holzem, 2014). Briefly, the standard method was followed, but the sample volumes were scaled down for a 96 well plate (from 2 mL to 200 µL). A standard curve was constructed by performing serial 50% dilutions on the stock ammonium sulfate solution (25 mg/L). Two hundred µL of each dilution was pipetted onto a 96 well plate. Two hundred µL of either synthetic wastewater (influent) or bioreactor effluent was also added to the 96 well plate in triplicate. A phenol solution and a nitroprusside solution were added. After one h of incubation, each well was colorimetrically read at 620 nm using a Thermo Electron Corporation Multiskan MCC (Waltham, MA) plate reader. Ammonium concentrations in each sample were calculated based on the standard curve (R^2 = 0.9839 for concentrations ranging from 0 to 25 mg/L NH_3-N).

Nitrite was measured using a modified Griess method (Holzem, 2014). Briefly, 50 µL of sample or standard were added to a 96 well plate. Fifty µL of 1% sulfanilamide in 3N HCl was added to each well. After a ten min incubation at room temperature, 50

µL of 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride was added to each well and incubated at room temperature for 10 min. Absorbance was measured at 540 nm on a Thermo Electron Corporation Multiskan MCC (Waltham, MA) plate reader. Nitrite concentrations in each sample were calculated based on the standard curve (R^2 = 0.9969 for concentrations ranging from 0 to 345 mg/L NO_2^- - N).

To measure nitrate, all samples underwent a cadmium reduction in order to convert nitrate to nitrite (Tu, 2010). Then, samples proceeded to the nitrite colorimetric method. Once concentration values were obtained, the original nitrite concentration was subtracted to give the nitrate concentration. Nitrate concentrations in each sample were calculated based on the standard curve (R^2 = 0.9942 for concentrations ranging from 0 to 722 mg/L NO_3^-).

Phosphate was measured using a microplate method (Hernandez, 2003). Briefly, a reactive solution was made fresh each sampling day consisting of 0.6% ammonium heptamolybdate, 12.75% sulfuric acid, 1.08% ascorbic acid and 0.0163% antimony potassium tartrate. Two hundred and fifty µL of either sample or standard was added to a 96 well plate and 30 µL of reactive solution was added to each well. The mixture was incubated for 10 min at room temperature and the absorbance was measured at 655 nm. Phosphate concentrations in each sample were calculated based on the standard curve (R^2 = 0.9953 for concentrations ranging from 0 to 570 mg/L PO_4-P).

Microbial Sampling and DNA Extraction. After steady state was reached in each phase, microbial community samples were collected at each sample port as well as the

top of the reactor. Samples were frozen at -20 ^{o}C, prior to DNA isolation. For each extraction, 1.5 g of cocopeat was added to a sterile 1.5 mL microfuge tube with 200 µL of breaking buffer (2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 100 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0), 200 µL of phenol:chloroform:IAA, and 0.3 g of 450 – 600 µm glass beads. Samples were vortexed at full speed for 5 min. Then, 200 µL TE buffer was added and mixed. After centrifugation at 13,000×g for 5 min, the top layer was collected into a clean microfuge tube. One mL of phenol:chloroform:IAA was added. After vortexing for 1 min and centrifuging for 1 min, the top layer was collected and another round of phenol:chloroform:IAA purification was performed. Afterwards, 1 mL of chloroform:IAA was added, vortexed and centrifuged for 1 min. After collecting the top layer into a new tube, 1 mL of 190 proof ethanol was added and centrifuged at 13,000×g for 3 min. The supernatant was decanted. The pellet was resuspended in 200 µL of TE buffer and 15 µL RNAase A and incubated at 37 ^{o}C for 1 h. Twenty µL of 3M sodium acetate and 0.5 mL of 190 proof ethanol was added and tubes were inverted to mix. After centrifugation at 13,000×g for 3 min, the supernatant was removed and 200 µL of ice cold 70% ethanol was added. Tubes were centrifuged at 13,000×g for 3 min, the supernatant removed, and DNA pellets were dried at 37 ^{o}C. DNA was resuspended in 15 µL of sterile deionized water.

The phenol/chloroform/isoamyl alcohol mixture was obtained from Life Technologies (Carlsbad, CA). Concentration and purity of DNA was measured on a Nanodrop spectrophotometer (Thermo Fisher Scientific). Only samples with high quality DNA were used for subsequent analysis.

T-RFLP. PCR was performed targeting the *16S* gene, with the 6 – carboxyfluorescein-labeled fluorescent forward primer (27F) and reverse primer 1392R. PCR conditions used were 94 ºC for five min followed by 35 cycles of 30 s at 94 ºC, 30 s at 50 ºC, and 30 s at 72 ºC, with a dissociation step at the end for quality control. Amplicons were purified utilizing a Qiagen PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Final PCR product concentrations and purity were measured on a Nanodrop spectrophotometer (Thermo Fisher Scientific). One hundred ng of purified PCR product and 10 U of *Msp1* (New England Biolabs, Beverly, MA, USA) were used for each T-RFLP reaction. The mixture was incubated at 37 ºC for 2 h. Analysis of fragments was performed as previously described in Chapter 4. Applied Biosystems GeneScan v3.7.1 software (Foster City, CA) was utilized to visualize T-RFLP profiles. T-REX online (Culman et al., 2009) software were used. Nonmetric multidimensional scaling (nm-MDS) analysis was performed and ordination plots are generated.

Statistical Analysis. The unpaired, two tailed student's t test was used to identify statistical differences between samples. Results were considered statistically different when the p-value <0.05.

5.3 Results and Discussion

A summary of the nitrogen, phosphorus, BOD, DO, pH, TSS, and VSS data for both the influent wastewater and effluent from each bioreactor for all four phases is shown in Table 5.1.The average BOD of the synthetic wastewater was 294.5 $\pm$ 115.5 mg/L. The average effluent BOD from Celite®, cocopeat, and sphagnum peat was 5.9 $\pm$ 4.0, 3.1 $\pm$ 3.2, 2.6 $\pm$ 2.4 mg/L, respectively. These correspond to removal efficiencies of 98.0, 98.9, and 99.1% for Celite®, cocopeat, and sphagnum peat, respectively. Each packing medium had significantly lower effluent concentrations than the influent ($p<0.001$). The BOD removal was statistically identical between cocopeat and sphagnum peat ($p>0.05$). Celite® had statistically higher BOD concentrations throughout ($p<0.05$). These data suggest that cocopeat is a suitable packing material for supporting microbial growth.

The average DO of the influent wastewater was 1.3 $\pm$ 1.2 mg/L. The average effluent DO from Celite®, cocopeat, and sphagnum peat was 1.6 $\pm$ 1.0, 3.9 $\pm$ 1.6, 4.6 $\pm$ 1.7 mg/L, respectively. The effluent from the Celite® reactors was not statistically different than the influent ($p>0.05$). However, both the cocopeat and sphagnum peat bioreactors had statistically higher concentrations of DO in the effluent ($p = 7.32 \times 10^{-11}$, 1.41×10^{-12}, respectively). This could be because oxygen infiltrates more readily due to the porosity of these media. Sphagnum peat had statistically higher concentrations of DO than cocopeat ($p = 0.00083$).

The average pH of the influent wastewater was 7.70 ± 0.38. The average effluent pH from Celite®, cocopeat, and sphagnum peat was 6.36 ± 0.39, 4.18 ± 0.69, 3.95 ± 0.33, respectively. Effluent pH was significantly lower for all three packing media, however cocopeat and sphagnum peat were statistically lower than Celite® ($p<0.001$). This is probably because peat is known to be acidic (Shaw, 2012), unlike inert clay pellets such as Celite®. The effluent pH in the cocopeat reactor was statistically higher than in the sphagnum peat ($p = 0.025$), suggesting that cocopeat is less acidic than sphagnum peat. The decrease in pH in all three reactors is also likely linked to biological activity, specifically nitrification, which is known to produces protons, lowering pH (Xu, 2006).

Table 5.1a Influent wastewater characteristics.

	Influent	
	ALL	
	Ave	Std Dev
Ammonium (mg/L)	122	27.7
Nitrate (mg/L)	0.07	0.16
Nitrite (mg/L)	0.00	0.02
Phosphate (mg/L)	9.59	5.76
TSS (mg/L)	9.03	3.15
VSS (mg/L)	7.09	2.27
pH	7.7	0.38
DO (mg/L)	1.3	1.2
BOD (mg/L)	312	146

Table 5.1b Effluent wastewater characteristics for each media type.

	Celite							
	I		II		III		IV	
	Ave	Std Dev	Ave	Std Dev	Ave	Std Dev	Ave	Std Dev
Ammonium (mg/L)	61.9	4.3	61.8	4.2	53.7	6	58	25.2
Nitrate (mg/L)	33	12.3	12.3	6.36	6.71	6.76	2.86	5.7
Nitrite (mg/L)	4	4.6	1.81	1.05	0.2	0.4	0.39	0.55
Phosphate (mg/L)	8.1	4.3	10.3	1	8.79	1.83	7.78	1.31
TSS (mg/L)	8.5	1.3	13.8	6.6	6.13	1.92	4.95	1.19
VSS (mg/L)	7.1	1.1	12.1	5	6.06	0.97	4.34	1.5
pH	6.47	0.54	6.39	0.23	6.22	0.12	6.34	0.43
DO (mg/L)	2.4	0.9	1.6	1	0.8	0.22	1.7	0.97
BOD (mg/L)	6.88	4.02	4.2	2.8	8.1	3.9	5.6	4.18

	Cocopeat							
	I		II		III		IV	
	Ave	Std Dev	Ave	Std Dev	Ave	Std Dev	Ave	Std Dev
Ammonium (mg/L)	58.6	3.7	55	3.2	55.4	3.6	63.2	28.1
Nitrate (mg/L)	41.5	18.7	27	12.7	15.5	19	10.9	11.5
Nitrite (mg/L)	0.8	1.2	0.03	0.02	0	0	0	0.13
Phosphate (mg/L)	8.6	5.9	8.1	2.4	7.56	0.77	8.83	2.78
TSS (mg/L)	7	3.3	6.1	1.6	4.44	2.29	4.68	3.01
VSS (mg/L)	6	2.7	6	1.4	4.32	2.37	4.97	2.87
pH	5.21	0.69	3.99	0.24	4.26	0.19	3.71	0.41
DO (mg/L)	3.5	1.2	4	0.9	2.9	1.21	4.4	2.17
BOD (mg/L)	3.31	3.06	3.9	3.9	3.2	3.4	2.5	2.02

	Sphagnum Peat							
	I		II		III		IV	
	Ave	Std Dev	Ave	Std Dev	Ave	Std Dev	Ave	Std Dev
Ammonium (mg/L)	49.2	6.6	63	4.3	51.8	7.6	55.5	17.1
Nitrate (mg/L)	38.5	22.7	30.1	17	10.9	7.84	7.5	8.74
Nitrite (mg/L)	0	0	0.04	0.03	0.1	0.1	0	0.14
Phosphate (mg/L)	8.1	3.6	6.6	0.3	7.57	0.57	7.16	2.11
TSS (mg/L)	7.1	3.6	7.1	2.2	4.71	0.77	4	2.39
VSS (mg/L)	6.9	2.6	7	2.4	3.59	0.14	4.59	3.4
pH	4.17	0.21	4.2	0.14	3.98	0.12	3.59	0.23
DO (mg/L)	5.5	0.8	5.6	0.8	2.4	0.58	4.4	1.91
BOD (mg/L)	3.08	1.62	1.4	1.6	3.1	2.6	3.2	2.65

Nutrient removal. The average ammonium concentration of the influent wastewater was 121.6 $\pm$ 27.7 mg/L (Figure 5.3a). The average effluent ammonium concentrations for all four phases from Celite®, cocopeat, and sphagnum peat were 55.3 $\pm$ 17.3, 59.3 $\pm$ 20.9, and 54.6 $\pm$ 16.9 mg/L, respectively. Each packing medium accomplished significant removal of ammonium ($p = 2.59 \times 10^{-23}$, 1.14×10^{-17}, 1.30×10^{-17}, respectively). Overall, cocopeat had significantly higher concentrations of ammonium compared to Celite® ($p = 0.038$). However, cocopeat and sphagnum peat differences were marginally significant ($p = 0.063$). Our attempts to change the redox conditions in the reactors in the 4 experimental phases did not translate to significantly different ammonium removal for neither the Celite® nor cocopeat reactors ($p>0.05$). However, a significant reduction of ammonium was accomplished when comparing Phase I and the three remaining phases in the sphagnum peat reactors with a concentration of 73.97 $\pm$ 10.50 mg/L in Phase I and an average of 53.73 $\pm$ 13.63 mg/L during the three latter phases.

Nitrite concentrations were variable throughout the Phase I and II (Figure 5.3b). The average nitrite concentration in the influent was 0.002 $\pm$ 0.024 mg/L. Overall, Celite® had significantly higher concentrations of nitrite in the effluent ($p = 0.00038$). The presence of nitrite coupled with the disappearance of ammonium suggests that ammonium oxidizing bacteria, responsible for the first step of nitrification (i.e., transformation of ammonium to nitrite) were present in the bioreactors. However, overall, cocopeat and sphagnum peat did not have nitrite concentrations statistically different from the influent ($p>0.05$), suggesting nitrite oxidizing bacteria (i.e, capable of

converting nitrite to nitrate) were present and may have been more efficient in the cocopeat and sphagnum peat as compared to Celite®. Phase changes did not significantly affect nitrite concentrations in each packing media ($p>0.05$). In Phase I, there was no statistical difference between packing media ($p>0.05$). In Phase II, cocopeat ($p = 0.01$) and sphagnum peat ($p = 0.02$) had significantly lower concentrations of nitrite than Celite®. In Phase III, Celite® was not statistically different than cocopeat or sphagnum peat. In Phase IV, Celite® was statistically different than cocopeat ($p = 0.01$) and sphagnum peat ($p = 0.01$), but cocopeat and sphagnum peat were not statistically different from each other ($p>0.05$).

The average nitrate concentration in the influent was 0.10 ± 0.16 mg/L (Figure 5.3.c). In Phase I, the highest effluent concentrations were achieved (33.04 ± 12.33, 41.53 ± 18.66, 38.53 ± 22.66 mg/L for Celite®, cocopeat, and sphagnum peat, respectively). This result was expected as nitrate is produced by nitrite oxidizing bacteria and requires aerobic conditions which were maximized in Phase I. In subsequent phases, a decrease in effluent nitrate was observed. With the increased hydraulic residence time in Phases II and III, an increased anoxic zone was created enabling bacteria to use nitrate as their electron acceptor for metabolic activity, hence enabling denitrification (Rittman and McCarty, 2001). In Phase IV, the nitrate concentration in the effluent from Celite®, cocopeat and sphagnum peat was variable with averages of 2.86 ± 5.70, 10.87 ± 11.48, and 7.50 ± 8.74 mg/L, respectively. In this phase, cocopeat and sphagnum peat had marginally statically significant differences ($p=0.093$) but they were both statistically different than Celite ($p>0.05$). Overall (all phases), the cocopeat and sphagnum peat

packing media achieved higher amounts of nitrification compared to Celite® demonstrated by the significantly higher effluent nitrate concentrations ($p = 2.07x10^{-7}$, $1.24x10^{-4}$, respectively). Interestingly, however, cocopeat and sphagnum peat effluent concentrations were not statistically different from each other ($p>0.05$) suggesting that these packing media are comparable for nitrogen removal. A description of nitrogen removal in each phase and design implications are discussed below.

Nitrogen - Phase I. The concentration of ammonium for all three packing media dropped immediately after start up likely due to sorption (Witter et al, 1989). Ammonium concentration then increases and stabilizes following microbial establishment of ammonium oxidizing bacteria. Nitrite concentrations are low at the beginning of Phase I ,then increase and finally stabilize once steady state is reached suggesting that nitrite oxidizing bacteria are less efficient than ammonium oxidizing bacteria. Nitrite concentrations were found to be highest in the Celite® reactor. Nitrate concentrations started off low and increase steadily throughout Phase I, likely as a more robust nitrite oxidizing community was established in the bioreactors. In Phase I, nitrification was accomplished, however, denitrification was not accomplished suggesting that the oxygen concentration was too high and that the residence time was not long enough to promote an anoxic zone.

Nitrogen - Phase II. Ammonium concentration was relatively steady throughout Phase II for all packing media. Nitrite concentration was highest in the Celite® reactors

while full removal of nitrite was accomplished in the cocopeat and sphagnum peat reactors. Nitrate concentrations started high at the beginning of Phase II, but steadily decreased throughout Phase II suggesting denitrifying activity. These data suggest that both aerobic and anoxic conditions were promoted during Phase II. Note that the Celite® reactor had higher concentrations of nitrite and lower concentrations of nitrate, suggesting that nitrite oxidizing bacteria were not as active in the Celite® reactors compared to the peat reactors. These data suggest that sphagnum peat and cocopeat can support nitrification and denitrification.

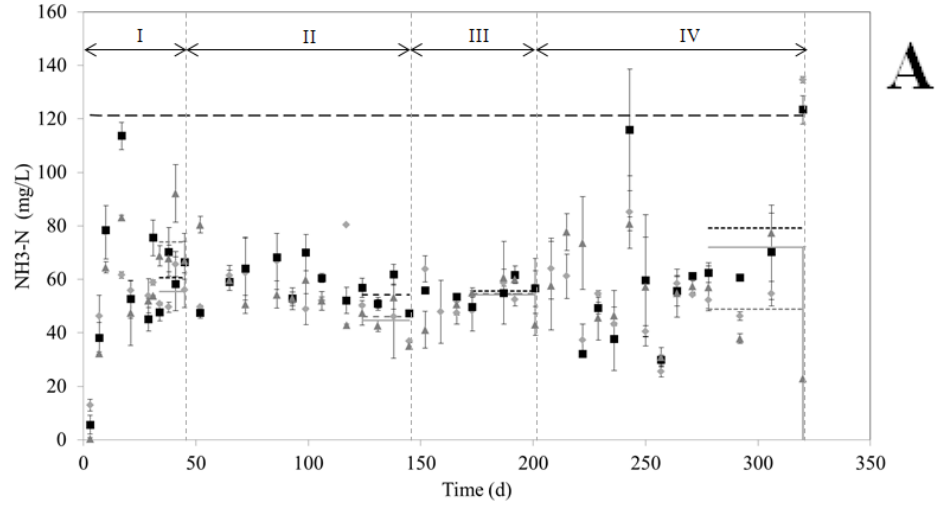
Nitrogen - Phase III. Ammonium concentrations remained steady throughout Phase III. Nitrite concentrations were low throughout phase III for all packing media. Nitrate steadily increases up to Day 42 and then decreases. This change in nitrate may be due to the re-establishment of the microbial community after the redox zone change. By the end of Phase III, nitrification and denitrification were observed in all packing media. However, no statistical difference between ammonium, nitrite, or nitrate concentrations were observed in any of the packing media types betweenPhases II and III. These data may suggest that the longer hydraulic residence time in this phase was not necessary.

Nitrogen - Phase IV. Throughout Phase IV, ammonium and nitrate concentration profiles were erratic in all bioreactors. This may be due in part to the steady decrease in pH in the cocopeat and sphagnum peat reactors and production of acid by nitrifiers (Xu, 2006). Peat is known to be acidic (Shaw, 2012), and it can be seen that from Phase I to

IV, pH decreased from 5.21 to 3.71 in the cocopeat and from 4.17 to 3.59 in the sphagnum peat. Nitrifiers and denitrifiers are sensitive to pH (Rittman and McCarty, 2001), so as the pH gradually decreases from Phase I to IV, nitrification and denitrification activity may have become stresses and led to the erratic ammonium and nitrate profiles observed. These data suggest that pH control may be needed for the long term operation of peat packed biofilters.

While cocopeat and sphagnum peat bioreactors achieved higher levels of nitrification than the Celite® bioreactors ($p=2.07 \times 10^{-7}$ and $p=1.24 \times 10^{-4}$ for cocopeat and sphagnum peat, respectively, as compared to Celite®), in no instance was full nitrogen removal achieved. This suggests that this reactor design could only achieve full nitrification /denitrification with lower influent nitrogen loadings. Furthermore, because nitrate was always present in the effluent, an anaerobic zone was never promoted which is required for biological phosphorus removal. The average phosphate concentration of the influent wastewater was 9.59 ± 5.79 mg/L (Figure 5.4). The average effluent phosphate concentration from the Celite®, cocopeat, and sphagnum peat bioreactors were 9.82 ± 4.43, 9.38 ± 6.01, and 8.09 ± 2.85 mg/L, respectively. None of the packing media accomplished statistically significant removal of phosphate ($p>0.05$). Phase changes did not affect phosphate removal for any of the packing media. This result is not unexpected as anaerobic treatment was not achieved as discussed above. Furthermore, both cocopeat and sphagnum peat were found to leach phosphate, evidenced by the occasional higher concentration of phosphate in the effluent as compared to the influent. To further

demonstrate the involvement of peat in phosphate release, jar tests experiments were performed and results showed that cocopeat released 46.5 $\pm$ 7.5 mg/L of phosphate per g of cocopeat and sphagnum peat released 141.5 $\pm$ 85.5 mg/L of phosphate per g of sphagnum peat. While problematic in this setup, if anaerobic treatment could be achieved, the additional phosphate may be able to be removed biologically. Additional experiments are needed to test this hypothesis.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NH3-N (mg/L)
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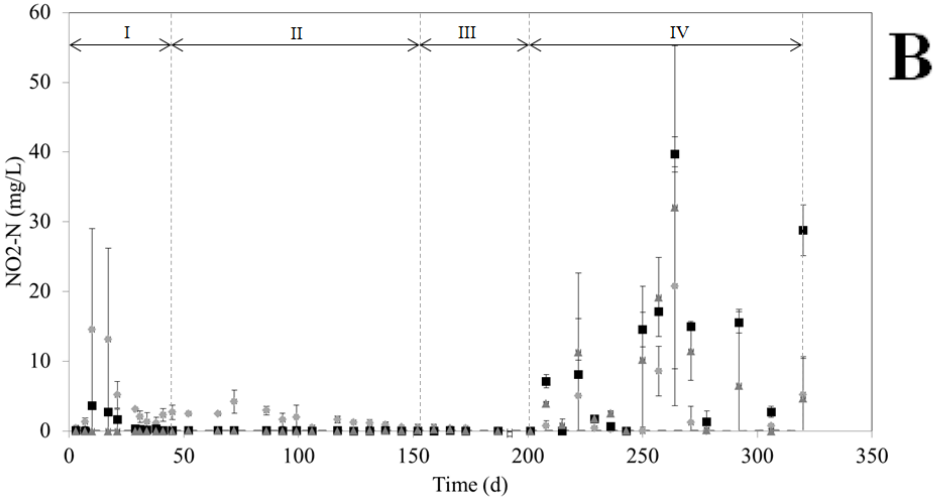
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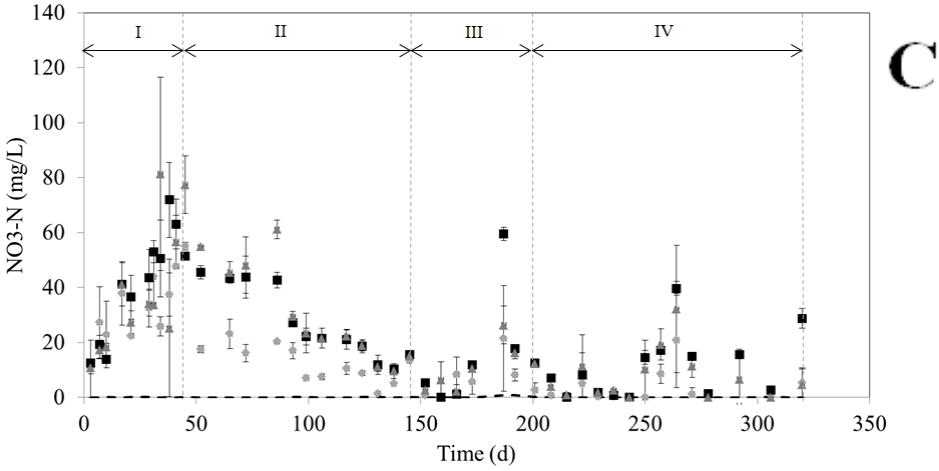
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Figure 5.3 A) Ammonium concentration, B) Nitrite concentration, and C) Nitrate concentration over time in influent (– –) and effluent of celite (◆), cocopeat (■), and sphagnum peat (▲) packed reactors. End of phase steady state ammonium concentrations are shown for celite (—), cocopeat (– – –), and sphagnum peat (– – –). Error bars represent the standard deviation of triplicate samples.

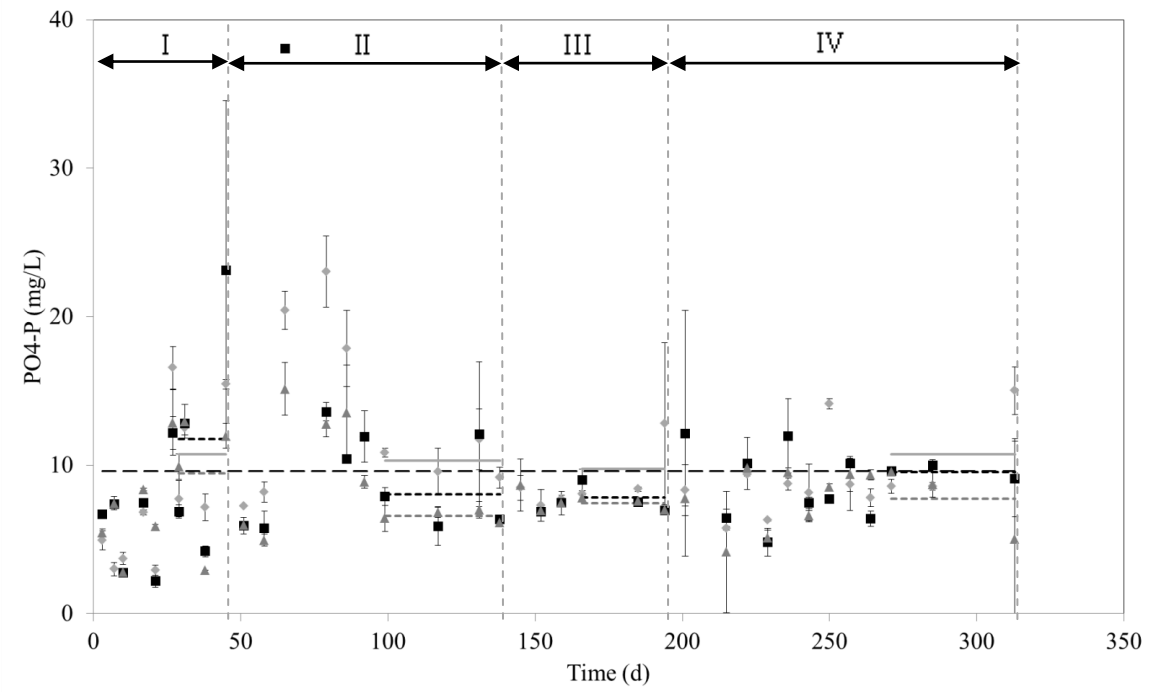

Figure 5.4 Phosphate concentration over time in influent (– –) and effluent of celite (◆), cocopeat (■), and sphagnum peat (▲) packed reactors. End of phase steady state ammonium concentrations are shown for celite (—), cocopeat (– – –), and sphagnum peat (– – –). Error bars represent the standard deviation of triplicate samples.

Microbial community distribution. There is an evident shift in communities of all packing media due to various factors, including ammonium, phosphate, and nitrate concentration (Figure 5.5). Throughout these shifts, communities from cocopeat and

sphagnum peat cluster together, indicating these two materials have similar physical-chemical properties that select for certain microbes, as compared to Celite®. More information can be obtained by separating these data by redox phases as discussed below.

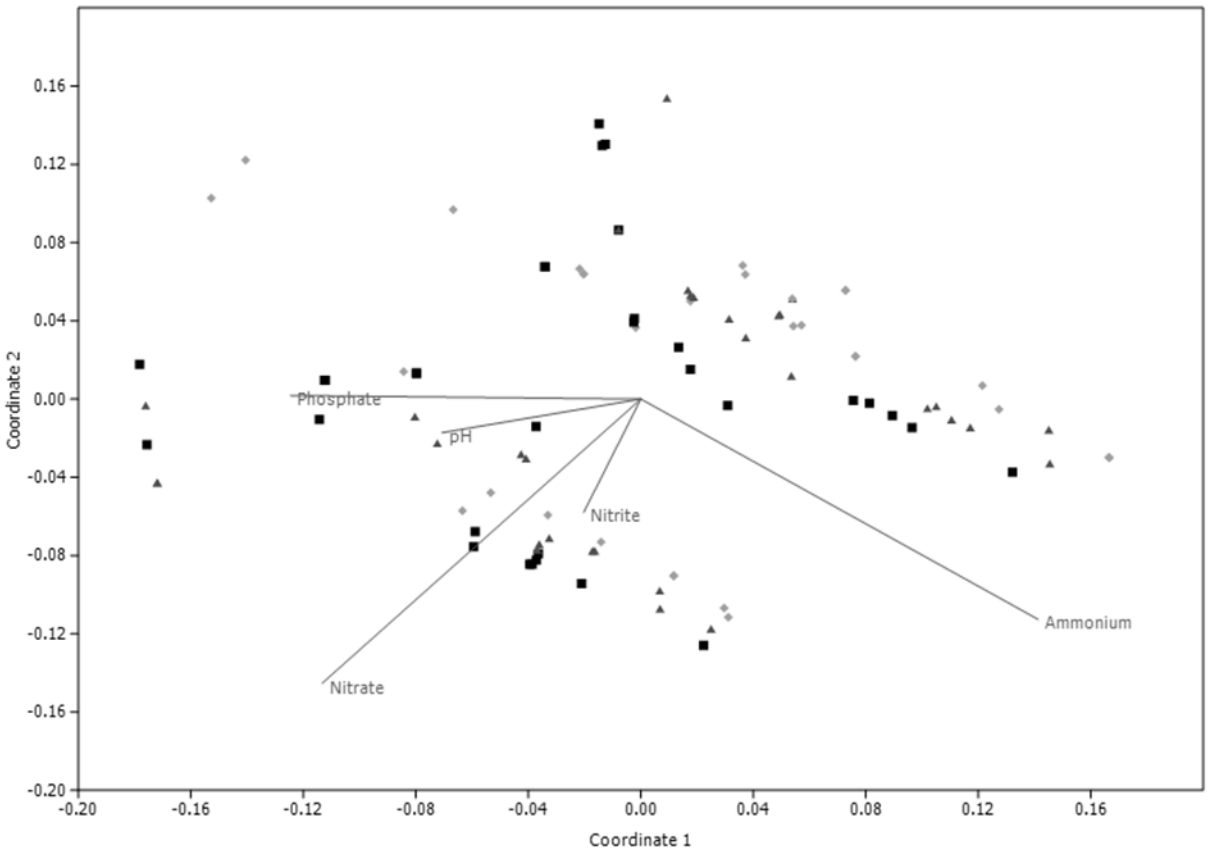

Figure 5.5 Non-metric multi-dimensional scaling (nm-MDS) of T-RFLP data of microbial community from celite (◆), cocopeat (■), and sphagnum peat (▲) packed reactors.

Phase I. Community differences were observed between the three media types in Phase I. Generally, cocopeat and sphagnum peat clustered around phosphate, nitrate, and ammonium, suggesting that these variables may have been important parameters impacting community structure differences. Celite® communities clustered around pH

and nitrite, indicating that these communities may have been controlled by different environmental factors than the communities in the cocopeat and sphagnum peat reactors. This is significant as peat may select for different microbial communities than an inert material such as Celite®, due to differences in surface area, porosity and nutrients.

Analyzing the nm-MDS plot shown in Figure 5.6 with PAST shows that in Phase I, ammonium concentration explains 93% of the microbial community differences between the three packing media and phosphate explains 2% (Figure B5). Using the coordinates generated by PAST for the nm-MDS plot, we can graph each nm-MDS component 1 and 2 with the ammonium and phosphate concentration, respectively, and we see that there is a linear relationship for each (R^2 = 0.9078 and 0.5604 for ammonium and phosphate, respectively). This maps well with the nutrient data from Phase I. Ammonium was important in Phase I due to the development of nitrification. Phosphate controlled microbial community distribution, likely due to the phosphate leaching in sphagnum peat and cocopeat.

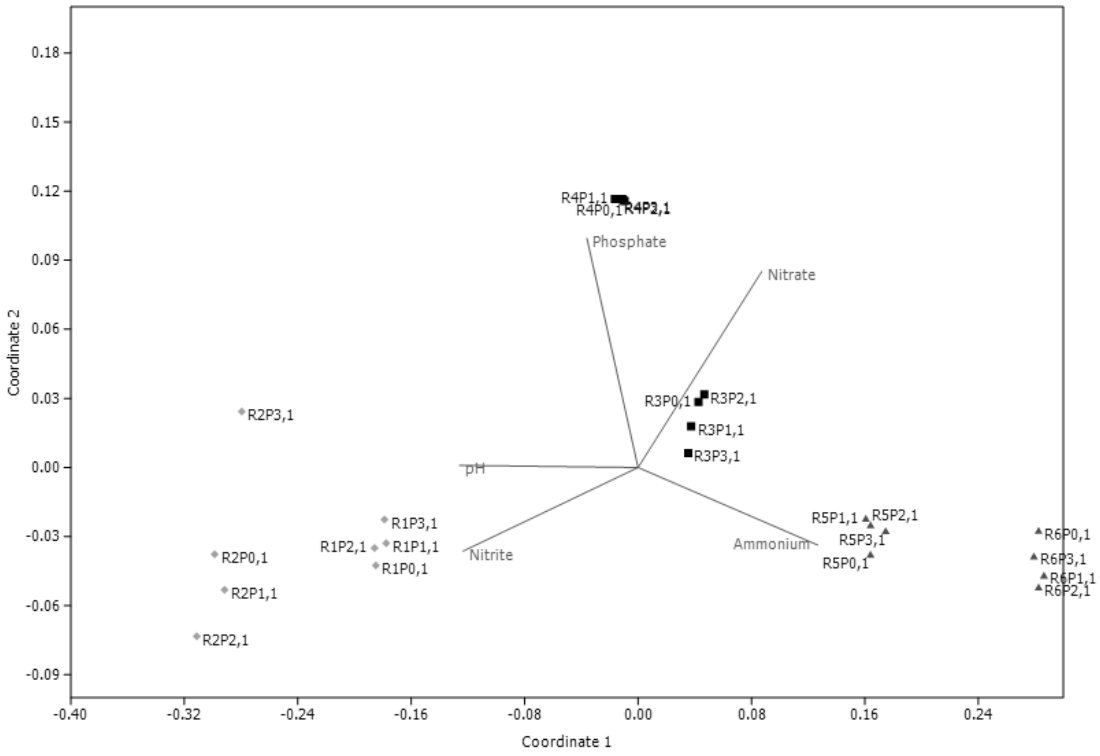

Figure 5.6. Nonmetric multidimensional scaling (nm-MDS) of T-RFLP data of microbial community from Celite® (◆), cocopeat (■), and sphagnum peat (▲) packed reactors during phase I.

Phase II. Cocopeat and sphagnum peat communities cluster along the nitrate axis. However, the community from one of the cocopeat duplicate reactors (reactor 4) was strongly impacted by phosphate. As in Phase I, the Celite® communities are strongly impacted by nitrite and pH. This may suggest that denitrification levels between the two peats and Celite® were important parameters controlling microbial community distribution.

Analyzing the nm-MDS plot shown in Figure 5.7 with PAST shows that in Phase II, nitrate concentration explains 59% of the microbial community differences and phosphate explains 16% (Figure B6). Using the coordinates generated by PAST for the nm-MDS plot, we can graph each nm-MDS component 1 and 2 with the nitrate and phosphate concentration, respectively, and we see that there is a linear relationship for each (R^2 = 0.8585 and 0.6513 for nitrate and phosphate, respectively). Nitrate was important in Phase II, likely due to the introduction of denitrifying organisms. Phosphate may have also been an important parameter controlling removal due to the effect of leaching.

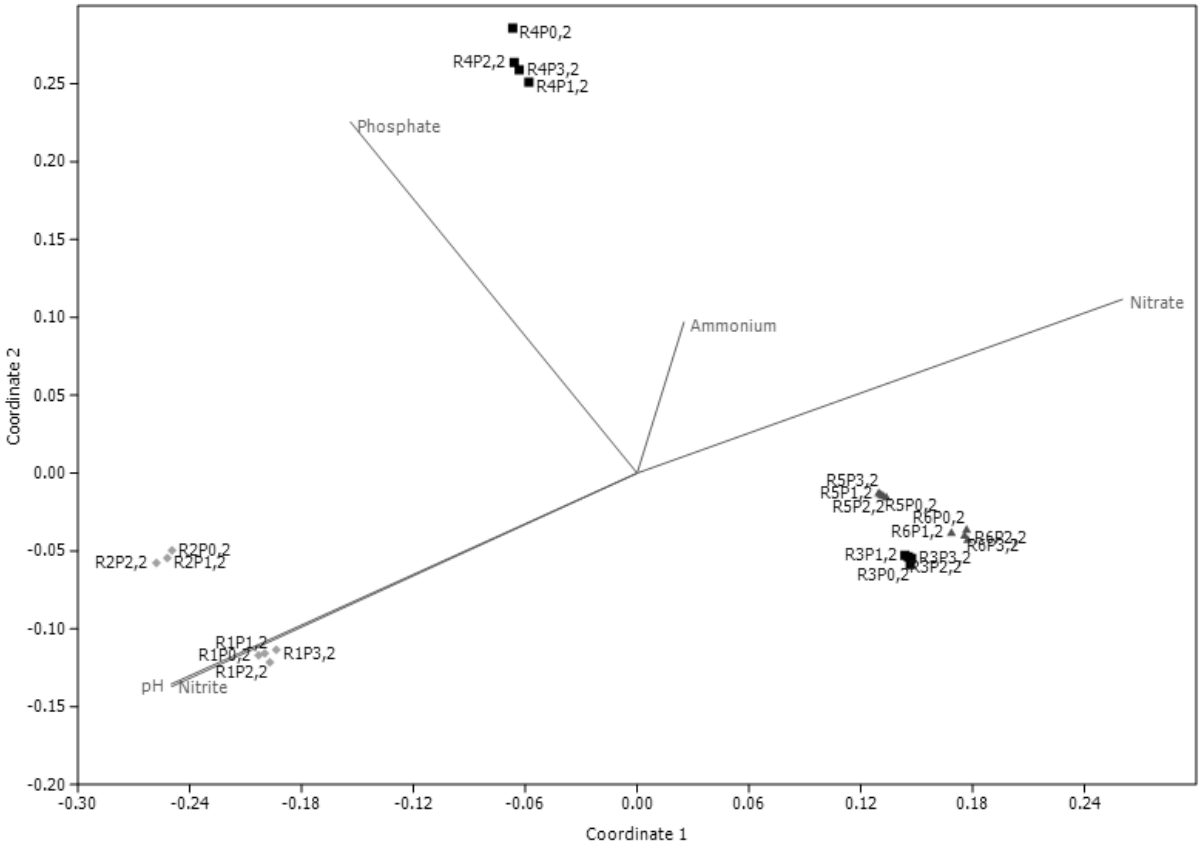

Figure 5.7. Nonmetric multidimensional scaling (nm-MDS) of T-RFLP data of microbial community from Celite® (◆), cocopeat (■), and sphagnum peat (▲) packed reactors during phase II.

Phase III. Cocopeat and sphagnum peat communities cluster around the nitrate and ammonium axes. The two Celite® reactors show distinct clusters indicating that the communities between the duplicate reactors were different.

Analyzing the nm-MDS plot shown in Figure 5.8 with PAST shows that in Phase III, ammonium concentration explains 56% of the microbial community differences and nitrate explains 43% (Figure B7). Using the coordinates generated by PAST for the nm-

MDS plot, we can graph each nm-MDS component 1 and 2 with the ammonium and nitrate concentration, respectively, and we see that there is a linear relationship for each (R^2 = 0.9875 and 0.9867 for ammonium and nitrate, respectively). Ammonium and nitrate may have been significant factors controlling microbial community distribution due to the effect of nitrification and denitrification. Phosphate may not have been as important in this phase, as compared to Phase III, because the effect of leaching may have been less significant. This result is consistend with data to be presented in Chapter 6 where phosphate leaching was found to no longer be significant after 110 d in field operated constructed wetlands.

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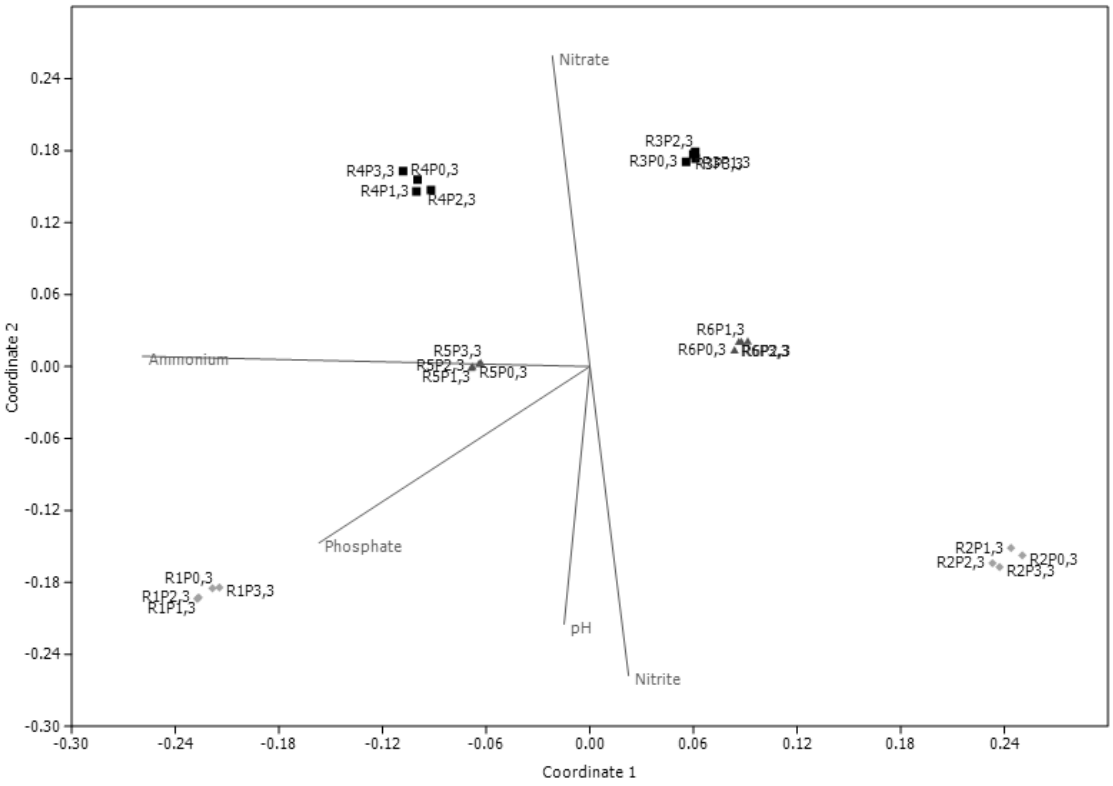

Figure 5.8. Nonmetric multidimensional scaling (nm-MDS) of T-RFLP data of microbial community from Celite® (◆), cocopeat (■), and sphagnum peat (▲) packed reactors during phase III.

Phase IV. Cocopeat communities clustered along the ammonium axis. Sphagnum peat communities clustered independently from the 5 environmental factors. Celite® communities clustered along the phosphate and nitrite axes.

Analyzing the nm-MDS plot shown in Figure 5.9 with PAST shows that in Phase IV, ammonium concentration explains 90% of the microbial community differences and nitrate explains 4% (Figure B8). Using the coordinates generated by PAST for the nm-MDS plot, we can graph each nm-MDS component 1 and 2 with the ammonium and

nitrate concentration, respectively, and we see that there is a linear relationship for each (R^2 = 0.9241 and 0.7422 for ammonium and nitrate, respectively). Ammonium and nitrate may have been significant factors controlling microbial community distribution due to the effect of nitrification and denitrification.

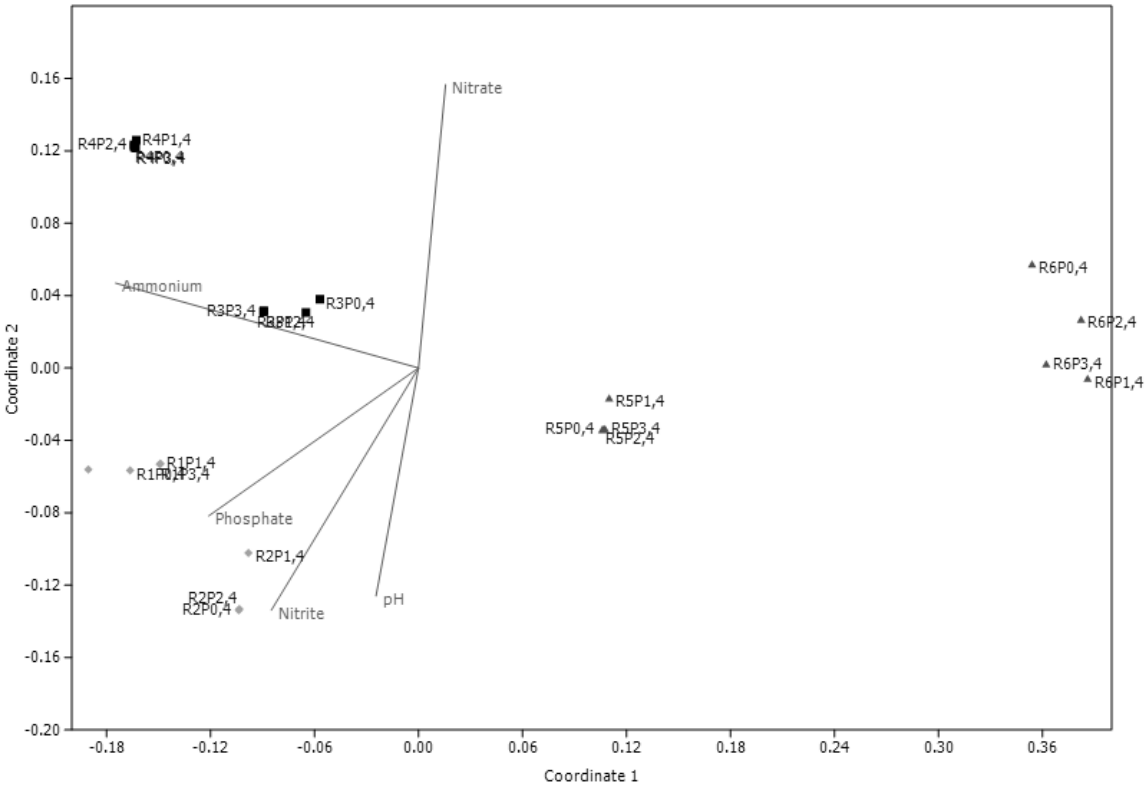

Figure 5.9. Nonmetric multidimensional scaling (nm-MDS) of T-RFLP data of microbial community from Celite® (♦), cocopeat (■), and sphagnum peat (▲) packed reactors during phase IV.

Overall, these nm-MDS data suggest that nitrification and denitrification were important parameters controlling microbial community distribution. This is expected as

these two processes are microbially driven. Additionally, phosphate was important during the first two phases, most likely due to the effect of phosphate leaching, but this was less significant in Phase III and IV.

5.4 Conclusions

In this study, 98.9% BOD and partial nitrogen treatment was obtained using cocopeat as a packing medium. The most efficient nitrification and denitrification was observed in Phase III. The deeper aerobic zone which was promoted with the increased hydraulic residence time allowed for further reduction of ammonium. Further work is needed to modify the design to promote anaerobic treatment to further reduce phosphorus effluent concentrations. Overall, cocopeat was found to be a comparable packing medium to sphagnum peat and provides an attractive alternative for field application. In particular, this packing material may be attractive in field applications where complete nutrient removal is not required such as aquaculture which is commonly employed in Vietnam where the effluent is discharged into fish ponds and the remaining wastewater nutrients act as fertilizer for duck weed, a food source for the fish.

In conclusion, cocopeat is a comparable packing media for biofilters compared to sphagnum peat, a traditional packing media. Cocopeat supports nitrification and denitrification of synthetic wastewater, but in this setup it did not accomplish phosphorus removal due to the short hydraulic residence time. Overall, cocopeat performs comparably to sphagnum peat, a well-documented traditional packing media for biofilters treating wastewater, and can achieve long-term operation.

Chapter 6. Vertical flow constructed wetlands packed with cocopeat for the removal of coliform bacteria and nutrients in septic tank effluent in Can Tho, Vietnam

6.1 Introduction

It is projected that the Millennium Development Goal target for sanitation will likely be missed. Over 1.8 billion people have gained access to improved sanitation since 1990, but there are still 2.5 billion people without access (WHO/UNICEF, 2014). Biofiltration by use of packed, fixed-film constructed wetlands is one wastewater treatment technology that has been well studied (e.g. Steer et al., 2002 [Ohio, USA]; Nerella et al., 2000 [Texas, USA]; Badkoubi et al., 1998 [Iran]; Haberl et al., 1995 [Europe]; Greenway and Simpson, 1996 [Australia]) and is being implemented in low-income countries to address this issue. Packed, fixed-film constructed wetlands are a feasible method of promoting wastewater treatment as they are relatively cheap to construct and operate, require limited maintenance, and tend to have high efficiencies of wastewater treatment, increased biological activity, anoxic soils, and sustain a rich diversity of biota due to the warm, tropical or subtropical climates (Kivaisi 2001, Saeed 2012, Fraser 2004). Naturally occurring media, such as peat, are common packing media for constructed wetlands and can be utilized where the attached growth, colonization and reproduction of microorganisms are promoted for the treatment of wastewater streams (Sherman, 2006). In order to minimize limiting factors such as cost and availability, locally-available packing media are ideal.

Cocopeat, a readily available product in Southeast Asia, is a by-product of coconut processing plants and comes from the outer husk of the coconut (Verdonck 1983), and unlike traditional peats such as sphagnum peat, cocopeat does not need to be mined. The coconut shell is shredded and then the fibers are removed. Cocopeat contains approximately 30% fibers and the remaining 70% is ground pith, which has a soil-like texture. In Chapter 5, it was shown that cocopeat can support nitrification, denitrification, and biological oxygen demand removal. In the current chapter, we perform a field evaluation of cocopeat as a biofilter packing medium.

The microbial activity in constructed wetlands is vital to achieving successful wastewater treatment. To optimize the removal of nitrogen and phosphorus in wastewater treatment, traditionally three redox zones are optimized: aerobic, anoxic and anaerobic (Zeng, 2010; Zhang, 2000). In order to degrade nitrogen, microbially driven nitrification and denitrification processes must occur (Vymazal 2007). These processes require aerobic and anoxic conditions, respectively (Chen et al, 1991; Li 2010; Adav 2009). Phosphorus transformations include a combination of physico-chemical processes (i.e., adsorption, desorption, precipitation, dissolution, fragmentation, leaching, sedimentation and burial) and biological processes (i.e., plant as well as microbial uptake and transformation) (Vymazal 2007). Microbial phosphorus removal occurs under anaerobic conditions (Delamenardiere, 1991; Brown, 2011; He, 2004; Converti, 1995). Phosphate accumulating organisms (PAOs) have a metabolic capacity that allows them to store large quantities of carbon at the expense of phosphate in anaerobic conditions and phosphate at the expense of carbon under aerobic conditions (Grady et al, 1999). These mechanisms

occur when certain heterotrophic bacteria sequester high levels of phosphorus as intracellular polyphosphate in the form of an energy storage material (Rittman and McCarty, 2001). When PAOs function optimally, the biomass contains 2 to 5 times the phosphorus content of normal biomass (Rittman and McCarty, 2001). Anaerobic conditions specially select for PAOs because most heterotrophic bacteria will not be able to oxidize organic matter during the residence time. PAOs will use phosphorus from the wastewater and store it in their cells as polyphosphate, a high energy compound (Grady et al, 1999). However, PAOs are not the only bacteria that uptake phosphorus albeit to a lesser degree. All bacterial cells are composed partially of phosphorus (Grady et al, 1999). In addition to microbial processes, plants are another significant sink of ammonium and phosphate in planted constructed wetlands where the nutrients serve as fertilizer.

Building on the operation of the cocopeat packed lab scale biofilters (Chapter 5), a pilot project was carried out in Vietnam to see if the bioreactors could be scaled up and attain simultaneous removal of nitrogen and phosphorous. The pilot project consisted of building and operating vertical flow constructed wetlands filled with cocopeat for the treatment of septic tank effluent from Can Tho University in the Mekong Delta of Vietnam. As with the lab scale reactors, the water level was controlled by the effluent pipe height in order to promote aerobic, anoxic and anaerobic redox zones to maximize the degradation and removal of nitrogen, phosphorus, and coliform bacteria.

6.2 Materials and Methods

Wetland Design and Operation. Five vertical flow constructed wetlands were built and operated in parallel at Can Tho University, in the Mekong Delta of Vietnam (Figure 6.1). Operation began in December 2012, during the beginning of the dry season, and continued to June 2013, well into the wet season. Each wetland was packed with a different ratio of cocopeat, the packing medium of interest, to gravel, a traditional packing medium, in order to test the optimal ratio of cocopeat to gravel for the treatment of septic tank effluent. Gravel was used in order to reduce the amount of phosphate leaching, as observed in the lab study (Chapter 5). A ratio was utilized because cocopeat is known to leach phosphorous which could lead to treatment issues (Chapter 5). The surface area for each wetland was 1.8 m x 0.9 m with a depth of 0.8 m. Using a water level control arm, the height of the effluent pipe was adjusted so that half of the wetland depth (0.4 m) was saturated in order to promote the anoxic and anaerobic zones thereby increasing the hydraulic residence time (Figure 6.2). Others have reported that the depth of the aerobic zone of a flooded or saturated soil is usually less than 1 cm (Vymazal, 2007). Each reactor was charged once daily with 56 L of septic tank effluent according to a previously published study (Gustofson, 2002). The charge was applied at a flow rate of 2 L/min. Twenty *Canna indica* were planted in each wetland at the beginning of the pilot project.

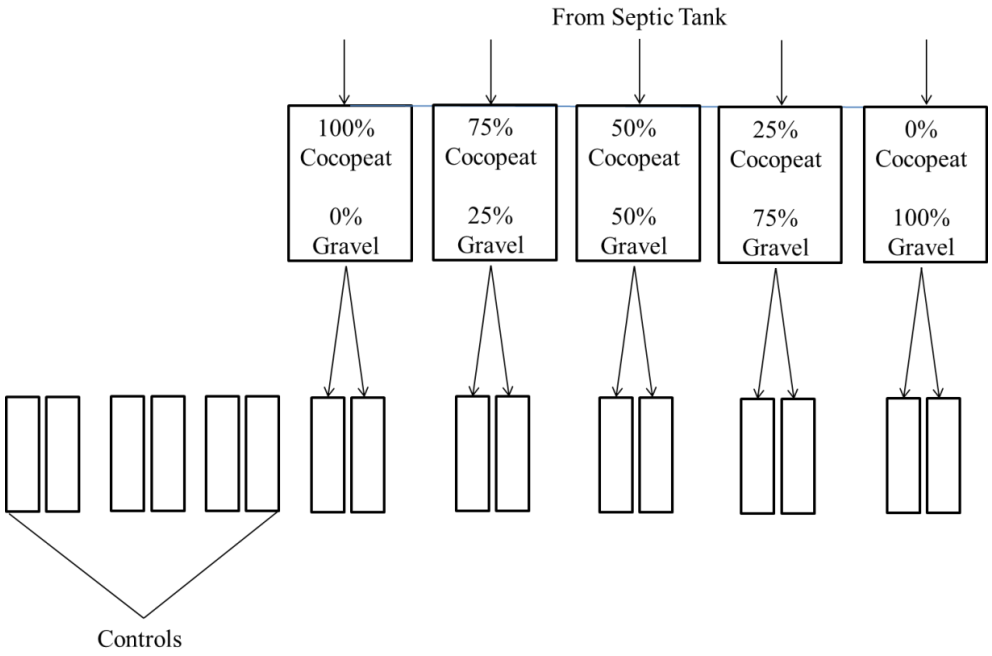

Figure 6.1a Schematic of overall system

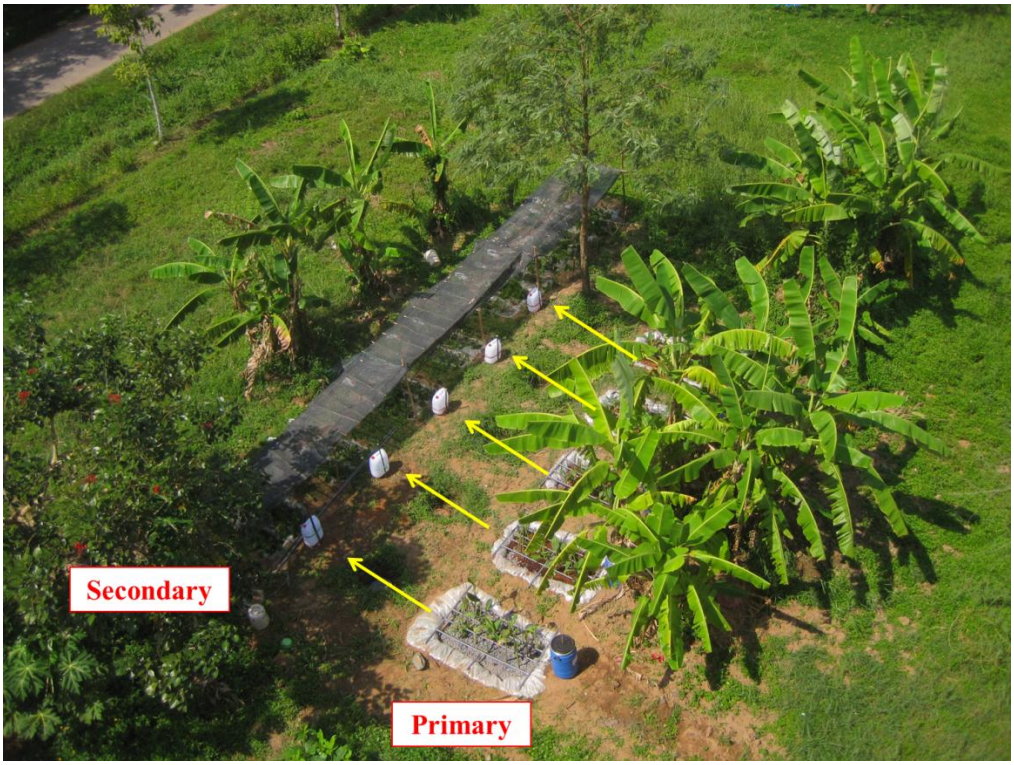

Figure 6.1b Aerial view of constructed wetlands (lower right) and ornamental planters (upper left)

As a secondary treatment step, effluent from the constructed wetlands was charged to smaller, horizontal flow constructed wetlands filled with gravel. These ornamental planters were operated in duplicate. Each planter was seeded with five *Caladium bicolor* ornamental plants, and duplicate planters received the effluent from one of the constructed wetlands. The effluent from each constructed wetland was split equally between two planters, operating in parallel. Three control systems were also operated in parallel, which receive septic tank effluent, tap water, and no water, respectively. For Control 1, duplicate planters were charged with raw septic tank effluent to determine the effect of untreated water on treatment efficiency as well as plant

biomass, as compared to effluent from the constructed wetlands. For Control 2, duplicate planters were charged with tap water to compare the effect of high nutrient (constructed wetland effluent) versus low nutrient (tap water) water on plant biomass. Finally, Control 3 involved duplicate planters that were not watered. This control was used to observe the effect of rain events on plant biomass. Each planter was charged once daily with a flow rate of 1 L/min, over a 30 min discrete charge.

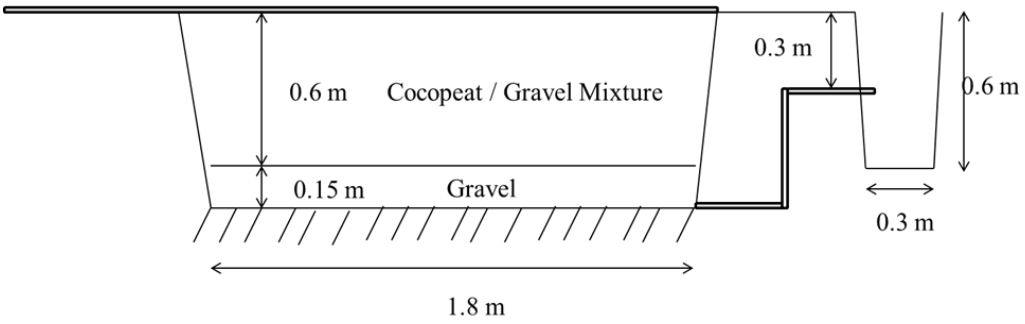

Figure 6.2 Cross-section of the constructed wetlands.

Sampling. The wetlands were operated for 26 weeks and 10 distinct time points were sampled. At each sampling point, influent wastewater was sampled directly from the septic tank. Primary effluent from each constructed wetland was collected from the water level control arm. Secondary effluent was also collected from the effluent tube of each ornamental planter. Water samples were collected in whirlpak bags (VWR, Radnor, PA) and tested immediately for ammonium, nitrate, nitrite, phosphate, biological oxygen demand (BOD), total suspended solids (TSS), pH, dissolved oxygen (DO), and total

coliform. All measurements were carried out in the Environmental Science Water Quality Laboratory at Can Tho University's College of Environment and Natural Resources as described below. Finally, 1 cm^3 of peat was collected in triplicate at 5 different depths (i.e., surface, 0.153 m, 0.28 m, 0.33 m, and 0.55 m) from each wetland for microbial community analysis. .

Water Quality Measurements. Total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods (IWA, 1999). Briefly, 0.2 µm glass microfiber filters (VWR, Radnor, PA) were prepared by rinsing with 20 mL of deionized water three times. Then, filters were incubated at 105 ºC and 550 ºC (Memmert, Eagle, WI) to remove any dust or other particles. Filters were weighed (Sartorius, Novi Sad, Serbia) to determine initial mass. For each filter, 500 mL of water was vacuum filtered. Then, the filters were incubated at 105 ºC for 1 h. Filters were weighed to determine TSS. Next, filters were incubated at 550 ºC for 1 h and then weighed to determine VSS. The TSS and VSS for synthetic wastewater and each effluent were measured in duplicate.

Total coliform was measured using a modification to the standard method for membrane filtration technique, as described in Chapter 3. Briefly, sufficient powdered growth medium to make 40 mL of M-Endo broth was initially aliquoted into sterile 50 mL plastic tubes. Deionized water was filtered through 0.2 µm mixed cellulose ester filters (Millipore, Billerica, MA) and added to the tubes to a final volume of 40 mL. Plates were prepared by pipetting 2 mL of broth onto a pad. Water samples were filtered

through 0.45 µm mixed cellulose ether filters (Whatman, Piscataway, NJ) using a vacuum pump. Filters were then placed on the medium-soaked pad and plates were incubated (Memmert, Eagle, WI) at 35 ºC for 24 h prior to enumeration.

BOD was measured as described in Chapter 5. Approximately 0.16 g of Hach nitrification inhibitor was added to each 300 mL bottle (Loveland, CO). Hach BOD nutrient buffer pillows were also added to each bottle (Loveland, CO). Bottles were filled to the neck and shaken to mix in the nitrification powder and the nutrient buffer. Then, the initial DO was recorded in duplicate using an Oakton DO 6 Acorn series meter (Thermo Fisher Scientific, Waltham, MA). Deionized water was added to fill the bottle and then a glass cap was tightly added. Bottles were incubated at 20 ºC (WTW, Weilheim, Germany) for 5 d. Then, bottles were uncapped and the DO was recorded immediately in duplicate.

DO was also measured the same meter used for BOD measurements. Wetland effluent was collected in a 300 mL BOD bottle and DO measurements taken immediately. pH was measured using an Orion 420A meter (Thermo Fisher Scientific, Waltham, MA). All measurements were taken in duplicate. Ammonium, nitrite, nitrate and phosphate were all measured using a Dionex ion chromatograph (Thermo Scientific, Waltham, MA) following standard methods (Method 9056a, US EPA). Detection limits for ammonium, nitrite, and nitrate was 0.1 mg/L as N. The detection limit for phosphate was 0.01 mg/L as P.

DNA isolations. DNA isolation was performed following the same protocol as described in Chapter 5.

T-RFLP. PCR was performed, targeting the 16S gene, with the 6 – carboxyfluorescein-labeled fluorescent forward primer (27F) and reverse primer 1392R. PCR conditions used were 94 ºC for 5 min followed by 45 cycles of 30 s at 94 ºC, 30 s at 50 ºC, and 30 s at 72 ºC, with a dissociation step at the end for quality control. Amplicons were purified utilizing a Qiagen PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Final PCR product concentrations and purity were measured on a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). One hundred ng of purified PCR product and 10 U of *Msp1* (New England Biolabs, Beverly, MA, USA) were used for each T-RFLP reaction. The mixture was incubated at 37 ºC for 2 h. Analysis of fragments was performed using an Applied Biosystems 3100 capillary sequencer (Foster City, CA) with POP6 polymer and ROX-labeled MapMarker 1000 size standards (Bioventures, Inc., Murfreesboro, TN) at the Duke University DNA Analysis Facility (Durham, NC). Standard procedures were followed. Applied Biosystems GeneScan v3.7.1 software (Foster City, CA) was utilized to visualize T-RFLP profiles. T-REX online software was used to process raw data through T-RF alignment to look at presence/absence of fragments (Culman et al., 2009). Any fragment 50 bp or smaller was excluded from data set to ensure no primer dimers were included in the analysis. PCA analysis was performed and ordination plots were generated using

Paleontological Statistics software (PAST) statistical software (Hammer, Ø. & Harper, D.A.T. 2006. Paleontological Data Analysis. Blackwell).

6.3 Results and Discussion

Coliform Removal. Coliform removal in the primary treatment (i.e., constructed wetlands) increased gradually over the period of the pilot test (Figure 6.3). At the initial sampling point (Day 45), coliform removal efficiencies were correlated to cocopeat volumetric loading in the constructed wetlands. The higher loadings experienced the highest removals while the no cocopeat treatment had the lowest (Figure 6.4). With time, the relationship between total coliform removal and volume of cocopeat was less significant. After Day 80, the effect of cocopeat volume on total coliform removal was not observed , removal efficiencies of total coliform for all constructed wetlands were approximately 3-log reduction, and there was no significant difference between treatments ($p>0.05$). At the end of the pilot test (Day 169), removal efficiencies were not significantly different between the different treatments ($p>0.05$) and each reactor achieved a 3-log reduction of total coliform, suggesting that over time the volumetric loading of cocopeat became less critical in terms of treatment performance. Microorganism fate within biofilters is not well established, although it is known that microorganisms can be either filtered out or captured either on or in a biofilm (Rittman and McCarty, 2001). Competition and predation also play a role in invading species (here, coliform) reduction (Rittman and McCarty, 2001). A possible explanation for this phenomenon is the increased physical/chemical attachment of peat materials as compared

to gravel. In addition, the rich organic content of the cocopeat may have supported biofilm growth more rapidly and helped filter out the coliform bacteria. We hypothesize that the cocopeat provides more surface area for biofilm to colonize on, as opposed to gravel, and this may have led to an increased biofilm development and corresponding amplified total coliform removal. Although the wetlands with higher gravel volumes experienced low total coliform removal rates at the beginning of the study, they reached 3-log removal by the end of the study, possibly due to the growing biofilm. The gravel medium takes longer for biofilm colonization as compared to cocopeat. This is likely to be partially due to differences in surface physico-chemical properties between cocopeat and gravel. It has been previously reported that the surface characteristics of bacteria and substrate are the determinant factors for the initial interaction and subsequent biofilm formation (Gallardo-Moreno et al, 2002). Mueller et al (2007) showed that there were clear differences in the genes required, mechanisms used, and biofilm phenotypes between *V. cholerae* biofilms on biotic versus abiotic surfaces. Since cocopeat is organic and gravel is inorganic, the resulting biofilms may have had very different characteristics which may account for the differences in coliform removal. However, while there were significant differences in primary treatment efficiency, when accounting for the secondary treatment, total coliform was greater than four log removal in all cases relative to the primary influent throughout the study period (Figure 6.4).

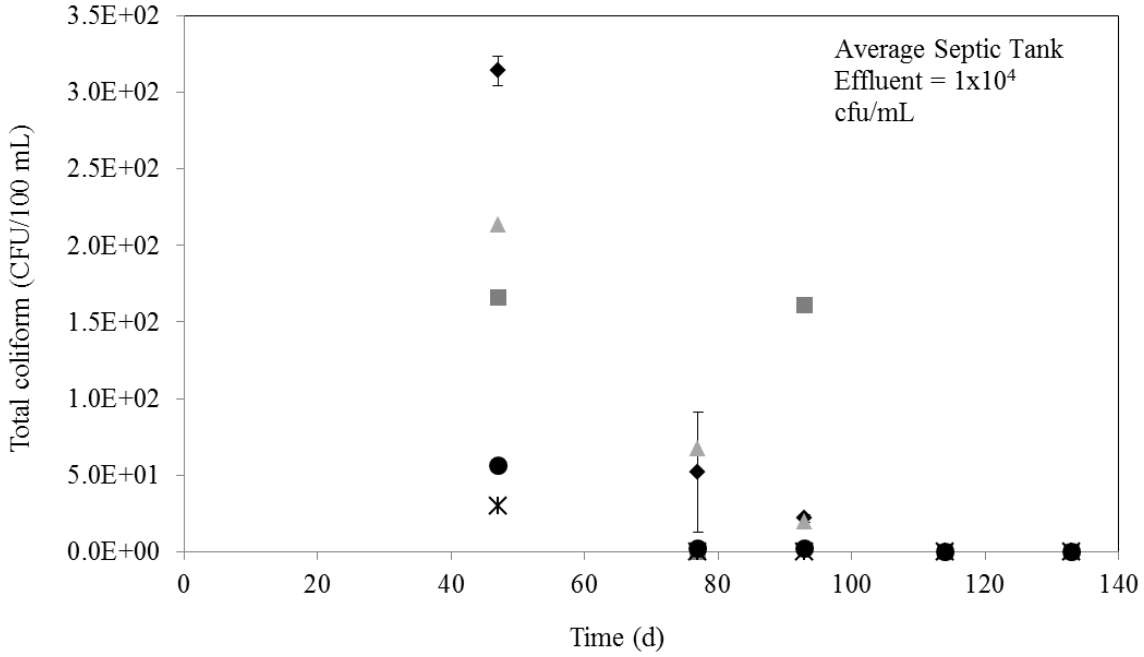

Figure 6.3 Total coliform concentration in the effluent of each constructed wetland over time. 0% cocopeat (◆), 25% cocopeat (■), 50% cocopeat (▲), 75% cocopeat (●), and 100% cocopeat (✶). Error bars represent the standard deviation of triplicate samples.

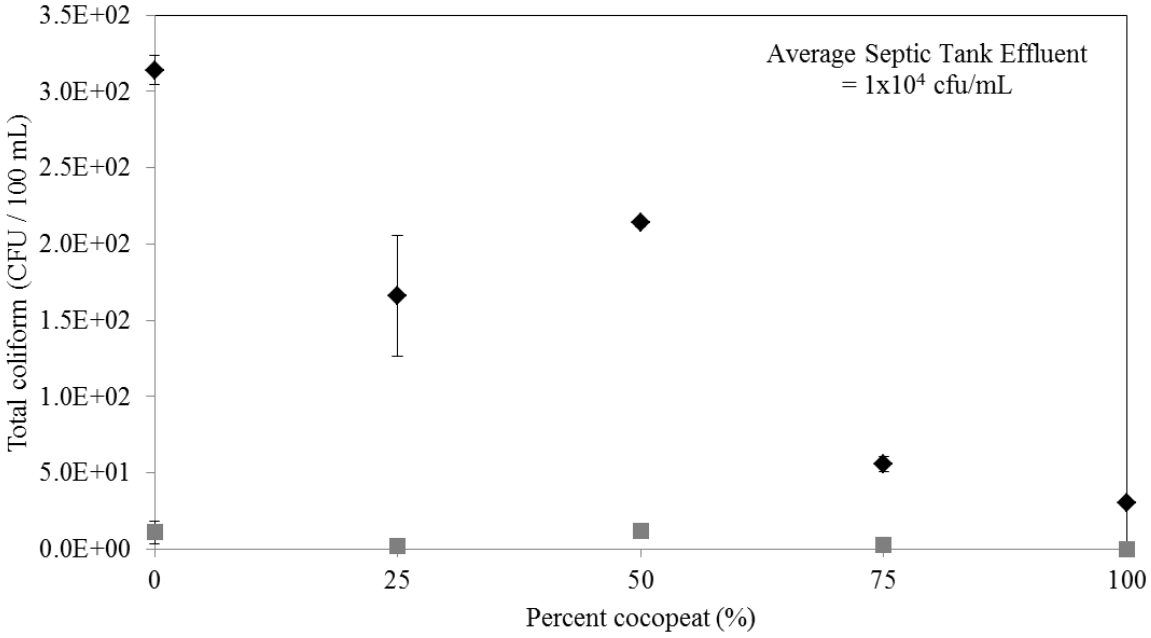

Figure 6.4 Total coliform removal at t = 49 d during primary (◆) and secondary (■) treatment. Error bars represent the standard deviation of triplicate samples.

Nutrient Removal. Phosphate profiles for the various treatments throughout the course of the study are shown in Figure 6.5. The average septic tank effluent phosphate concentration was 4.03 $\pm$ 0.68 mg/L. This compares well to the lab study (Chapter 5), as our phosphate concentrations were on the same order of magnitude (~9 mg/L). Steady state removal of phosphate removal was achieved within 110 days. At steady state, average effluent phosphate concentration were 0.44 $\pm$ 0.14, 0.36 $\pm$ 0.18, 0.69 $\pm$ 0.25, 0.83 $\pm$ 0.24, 1.32 $\pm$ 0.03 mg/L from the 0, 25, 50, 75, and 100% cocopeat wetlands, respectively. Phosphate concentration was observed to correlate with volumetric amount of cocopeat in the wetlands. However, over time, similarly to the coliform removal, this

relationship became less significant. Early on in the study (< 75 d), we observed leaching of phosphate from cocopeat, indicated by higher concentrations of phosphate in the effluent than in the influent. This result was not unexpected as phosphate leaching from cocopeat was also observed in the lab (Chapter 5). After 75 d for the three lowest cocopeat volumes and 100 d for all cocopeat volumes, the phosphate leaching became less significant as indicated by the net reduction of phosphate. At steady state (>110 d), all reactors had a phosphate removal greater than 70%. At steady state, each wetland had significantly lower phosphate concentrations than the influent ($p = 0.0001, 0.0004, 0.0006, 0.0008, 0.0005$, respectively) and the wetland with only cocopeat had statistically significant higher phosphate concentrations than the wetlands with 0, 25, and 50% cocopeat ($p = 0.0054, 0.0116, 0.27$ respectively), suggesting that at steady state, the removal of phosphorus correlated with the volume of cocopeat in each wetland (Figure 6.6). Interestingly, phosphate removal was accomplished in the wetlands, but not in the lab study previously described (Chapter 5). This may be in part because the ammonium and nitrate concentrations here were lower (discussed below), allowing a more substantial anaerobic zone to form. The reduction of nitrate may have been linked to the *Canna* planted in the constructed wetland. Nitrate is the most available nitrogen species available to plants (Xu et al, 2012; Casper et al, 1997), and following up on ammonium and nitrite oxidizing bacteria converting ammonium to nitrate, the plants may have taken up the nitrate, as well as the leftover ammonium, reducing the anoxic zone depth requirement for the full removal of nitrate. In the absence of oxygen and nitrate, an anaerobic zone will form, which may allow PAOs to reduce phosphate concentrations in

the water. Others have shown that when nitrate is present, the amount of fermentation that can occur is reduced (vital for PAOs) and allows heterotrophic bacteria to out-compete the PAOs for acetate, resulting in minimal rates of phosphate accumulation by microorganisms (Grady et al, 1999). Since the nitrate levels in this system were minimal, PAOs may be responsible for the phosphate reduction. Furthermore, the plants are likely partially responsible for the uptake of phosphate (Casper et al, 1997).

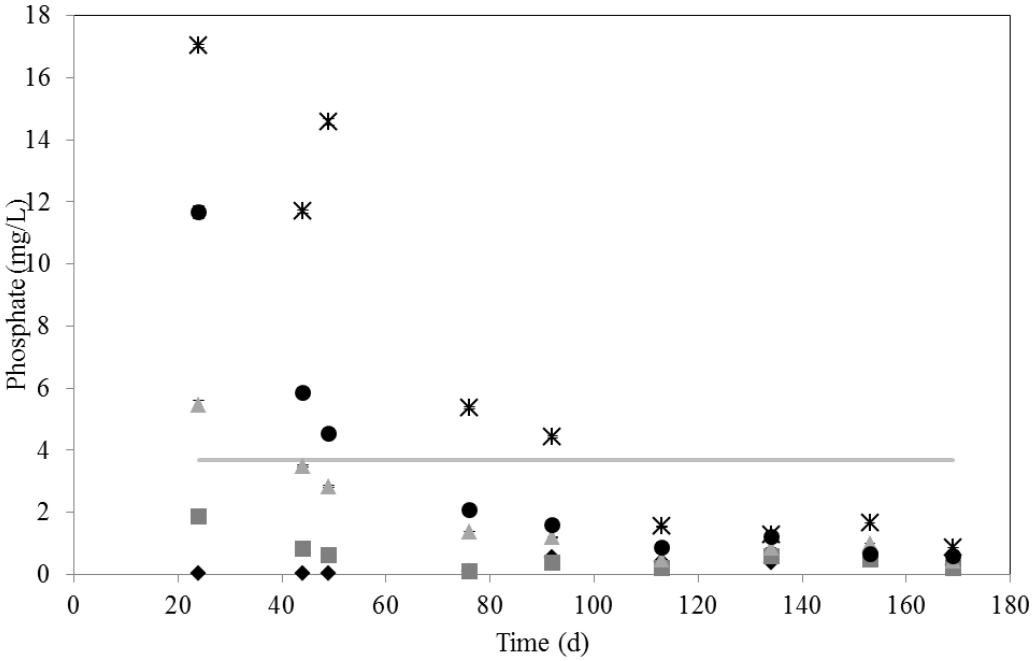

Figure 6.5 Phosphorus concentration in the effluent of each constructed wetland over time. 0% cocopeat (◆), 25% cocopeat (■), 50% cocopeat (▲), 75% cocopeat (●), and 100% cocopeat (✳). Average septic tank effluent concentration indicated by (▬). Error bars represent the standard deviation of triplicate samples.

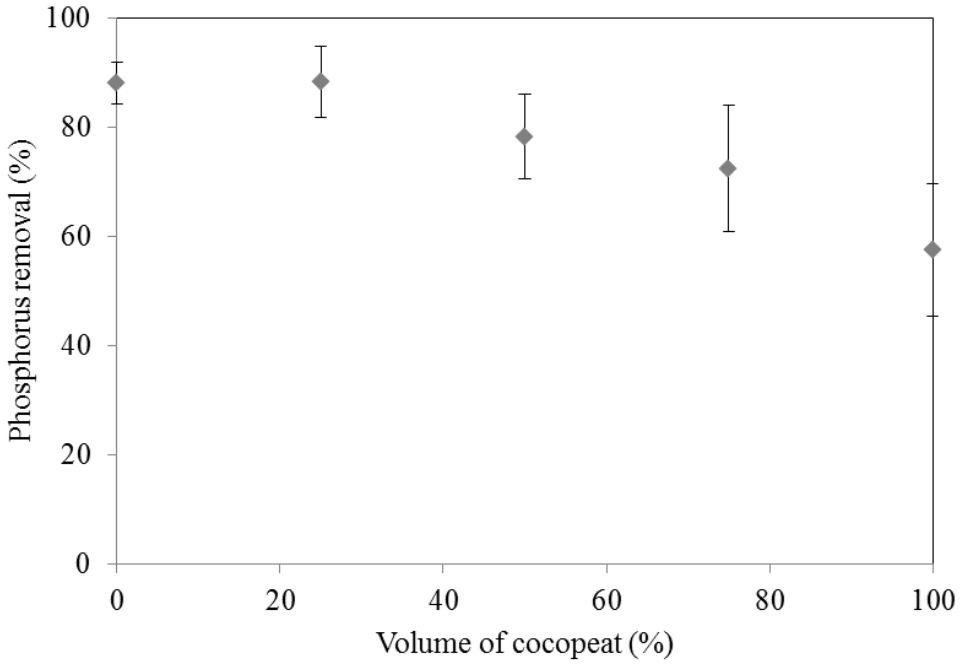

Figure 6.6 Phosphorus removal at steady state for each constructed wetland based on cocopeat percentage. Error bars represent the standard deviation of triplicate samples.

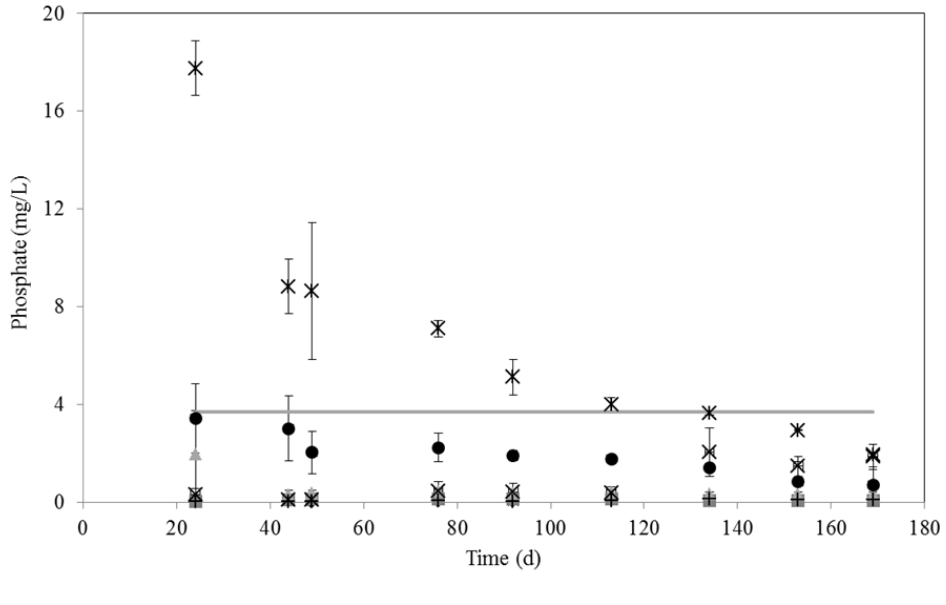

Figure 6.7 Phosphorus concentration in the secondary effluent of each constructed wetland over time. 0% cocopeat (♦), 25% cocopeat (■), 50% cocopeat (▲), 75% cocopeat (●), and 100% cocopeat (✶). Average septic tank effluent concentration indicated by (▬). The wastewater control (X) and tap water control (+) effluents are also shown. Error bars represent the standard deviation of triplicate samples.

Phosphate removal data for secondary treatment (gravel planters with *Caladium bicolor*) is shown in Figure 6.7. The average effluent from secondary treatment from the constructed wetland containing 0, 25, 50, 75, and 100% cocopeat were 0.07 $\pm$ 0.02, 0.09 $\pm$ 0.03, 0.37 $\pm$ 0.04, 1.19 $\pm$ 0.43, and 3.13 $\pm$ 0.78 mg/L, respectively. Steady state effluent concentrations from the controls were 1.45 $\pm$ 0.64 mg/L for the wastewater control and 0.106 $\pm$ 0.02 mg/L for the tap water control. Secondary effluent phosphate concentrations from the 0 and 25% cocopeat reactors were not statistically different ($p>0.05$), but secondary effluent from the 50 ($p=0.0028$), 75 ($p=0.0233$), and 100% ($p=0.007$) were

statistically different from the 0 and 25%. Interestingly, the 0 and 25% cocopeat secondary effluent were not statistically different than the effluent from the tap water control ($p>0.05$). Additionally, the 0 ($p=0.032$) and 25% cocopeat ($p=0.038$) had statistically different phosphate concentrations as compared to the septic tank effluent, but the 50, 75, and 100% cocopeat did not ($p>0.05$). At steady state, the secondary treatment step of gravel planters with *Caladium bicolor* plants accounted for additional removal of 80.7 $\pm$ 10.5, 66.9 $\pm$ 20.5, and 37.5 $\pm$ 25.6% of the 0, 25, and 50% cocopeat wetlands respectively. No additional removal was observed after secondary treatment for the 75 and 100% cocopeat effluents. At steady state, the control planters were able to remove 60.8 $\pm$ 17.2% of the phosphate from raw wastewater, and no change was observed in the phosphate concentrations of the tap water control. The additional phosphate removal accomplished during secondary treatment can be attributed to biomass uptake (Vymazal, 2007).

Ammonium concentration throughout the course of the study is shown in Figure 6.8. The average influent ammonium concentration was 58.7 $\pm$ 22.8 mg/L. This was lower than the concentration used in the laboratory experiments (120 mg/L) (Chapter 5). The average primary effluent ammonium concentration were 3.6 $\pm$ 2.4, 1.2 $\pm$ 0.57, 0.98 $\pm$ 0.61, 1.2 $\pm$ 0.91, 0.55 $\pm$ 0.41 mg/L from the 0, 25, 50, 75, and 100% cocopeat wetlands, respectively. Ammonium removal was greater than 95% in each wetland containing cocopeat throughout the course of study, suggesting ammonium removal reached steady state very early on during the operation, unlike phosphate removal. Throughout the course of study, ammonium removal was greater than 80% in the wetland containing

gravel only, and never reached the removal efficiency levels of wetlands with cocopeat. At steady state, the wetland containing only gravel had statistically lower ammonium removal as compared to the wetlands containing 25, 50, 75, and 100% cocopeat ($p = 0.0124$, 0.0097, 0.0064, and 0.0094, respectively). These data suggest that cocopeat facilitates nitrification. Nitrate and nitrite concentrations were below the detection limit in each wetland effluent throughout the course of the study. These data suggest that cocopeat in the wetlands supported denitrification. However, it is known that nitrate is the nitrogen species most available to plants (Xu et al, 2012; Casper et al, 1997), so it is possible that the *Canna* consumed the nitrate, reducing the need for an anoxic zone and denitrification.

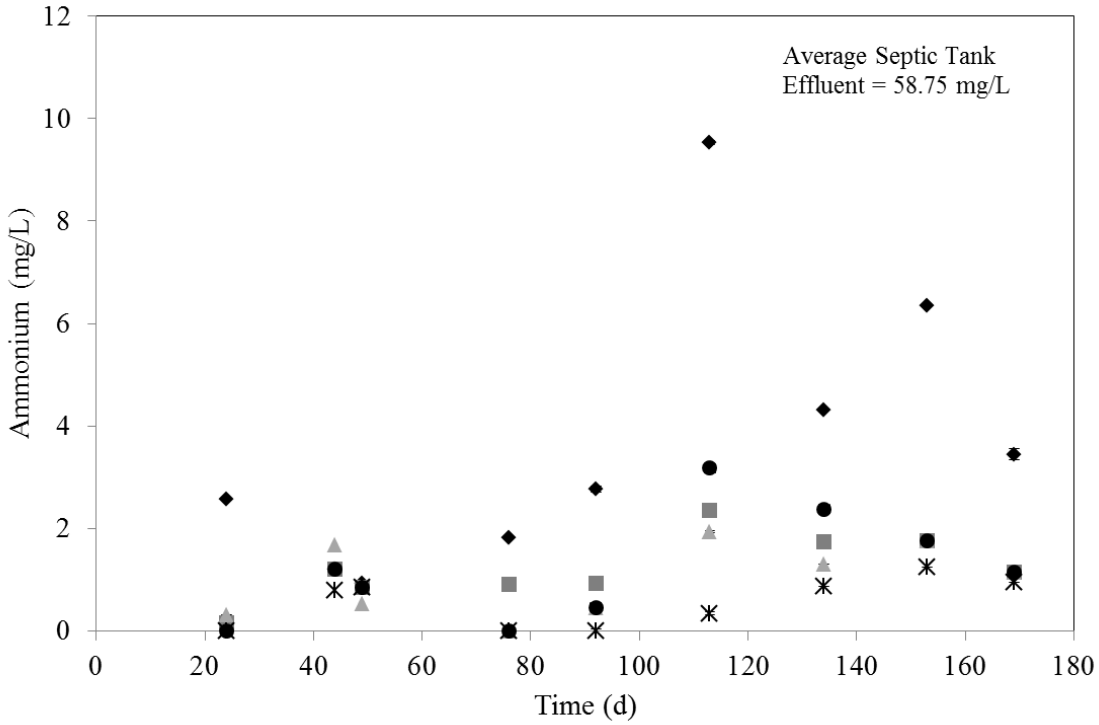

Figure 6.8 Ammonium concentration in effluent over time. 0% cocopeat (◆), 25% cocopeat (■), 50% cocopeat (▲), 75% cocopeat (●), and 100% cocopeat (✳). Error bars represent the standard deviation of triplicate samples.

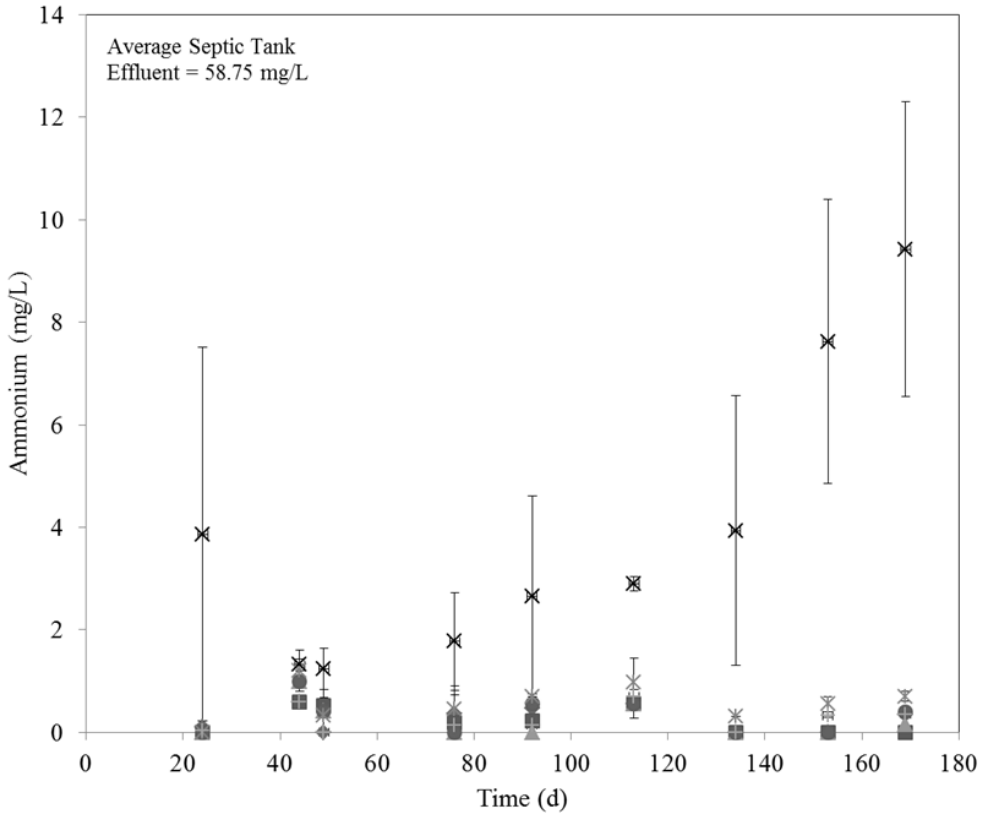

Figure 6.9 Ammonium concentration in secondary effluent over time. 0% cocopeat (◆), 25% cocopeat (■), 50% cocopeat (▲), 75% cocopeat (●), and 100% cocopeat (✳). The wastewater control (X) and tap water control (+) effluents are also shown. Error bars represent the standard deviation of duplicate reactors.

Ammonium removal data for secondary treatment is shown in Figure 6.9. The average effluent from secondary treatment from the constructed wetland containing 0, 25, 50, 75, and 100% cocopeat were 0.27 $\pm$ 0.23, 0.23 $\pm$ 0.24, 0.24 $\pm$ 0.33, 0.33 $\pm$ 0.33, and 0.58 $\pm$ 0.34 mg/L, respectively. Steady state effluent concentrations from the controls were 3.86 $\pm$ 2.69 mg/L for the wastewater control and 0.25 $\pm$ 0.24 mg/L for the tap water control. Secondary effluent from the constructed wetlands containing 0, 25, 50, and 75%

cocopeat were not statistically different from each other or the tap water ($p>0.05$). They were statistically lower than the secondary effluent from the wetland with 100% cocopeat ($p<0.05$), as well as the wastewater control ($p<0.05$). The secondary effluent from the 100% cocopeat wetland was statistically higher in ammonium than the other wetlands ($p>0.05$), and the tap water control ($p>0.05$), but was significantly lower than the wastewater control ($p = 0.0417$). At steady state, this secondary treatment step of gravel planters with *Caladium bicolor* plants was able to accomplish additional removal of 96.0 $\pm$ 4.3, 94.1 $\pm$ 10.2, 89.6 $\pm$ 11.8, 86.9 $\pm$ 14.4, and 49.0 $\pm$ 16.4% of the 0, 25, 50, 75, and 100% cocopeat wetlands respectively. At steady state, the control planters were able to remove 89.8 $\pm$ 4.5% of the ammonium from raw wastewater, and no change was observed in the ammonium concentrations of the tap water control. The additional removal of ammonium can be contributed to plant uptake (Xu, 2012).

It is clear that packing media ratio impacted removal efficacy of nutrients and total coliform. This is most likely due to differences in surface area, porosity, and previously established microbial communities between cocopeat and gravel (discussed above with respect to total coliform removal).

Microbial Community Distribution. An analysis of the molecular diversity of total bacteria associated with the packed constructed wetlands was performed using terminal restriction fragment length polymorphism (T-RFLP) and the structures of these communities among five different depths and five different wetland compositions were compared. Overall community shifts for each wetland at each depth and time point is

shown in Figure 6.10. The wetlands with 0 and 25% cocopeat had distinct communities at time 1 due to total coliform at depth A, B, C, D, and E. At time 3, each reactor had similar communities at depth A, B, C, D, and E, indicating over time, the reactors stabilize and select for similar bacteria, independent of the packing medium or depth. Reactor 3, 4, and 5 stabilized faster than reactor 1 and 2 (attributed to the higher amount of cocopeat) at depth A, B, C, D, and E. Differences in reactor 4 and 5 between time point 2 and 3 can be accounted for by phosphate concentration at depth B, C, D, and E. At lower depths, the microbial community in reactor 5 is still experiencing the effect of phosphate at depth D and E.

Clear temporal shifts can be observed with most of the Day 49 and Day 169 communities clustering. There is also a fairly distinct shift correlated to phosphate between wetland with 100% cocopeat and the others, most likely due to the increased effect of phosphate leaching as cocopeat content increased. Ammonium did not have much of an effect on microbial community. Coliform was critical at Day 49 as compared to the other time points, but then becomes less important as time increases. More information can be obtained by separating these data by cocopeat loading and depth, and this is discussed below.

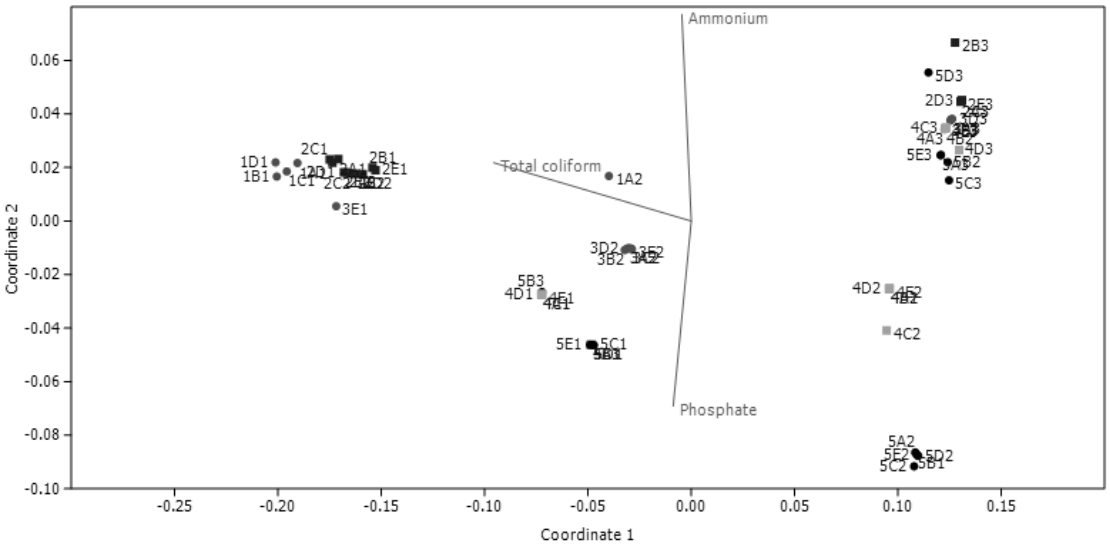

Figure 6.10 Each reactor at each depth and time point with three environmental factors: ammonium, phosphate, and total coliform. Data labeled wetland number, depth, and time point. Wetlands 1 – 5 were 0%, 25%, 50%, 75%, and 100% cocopeat, respectively. Depths A – E were surface, 0.153 m, 0.28 m, 0.33 m, and 0.55 m, respectively. Time 1 – 3 were 49, 92, and 169 d respectively.

Impacts of Cocopeat Loading. Differences were observed between the wetland with 25% and 100% cocopeat suggesting that the presence of higher amounts of cocopeat affects microbial community structure and supports additional treatment (Figure 6.11). Microbial community profiles in 25% cocopeat at Day 49 and 96 cluster suggesting coliform is an important parameter impacting microbial community shifts. The wetland with 100% cocopeat also clusters, however over time, the importance of microbial community distribution on phosphate and total coliform treatment efficiencies decrease as demonstrated by the tight clustering with the wetland with 25% cocopeat at Day 169. Over time, the community differences between the wetlands with 25 and 100% become

reduced as indicated by the cluster at Day 169. A few important conclusions can be drawn from these comparisons. First, environmental factors ammonium and phosphate have an opposite effect on microbial community. This result is expected as it is likely linked to the requirement of different redox conditions to support microbial communities at each depth. With an increase in cocopeat volume, there is a clear clustering by time, independent of depth. These data suggest there was no difference in redox zones, likely due to a shallow aerobic zone with a steep oxygen gradient leading to a large anoxic/anaerobic zone. This suggests these reactors could be designed to be shallower and possibly require less material. The effect of the *Canna* plants was important for this redox gradient, as in lab columns (Chapter 5) we were not able to accomplish an anaerobic zone. These differences in communities between the wetlands with 25 and 100% cocopeat can be attributed to differences in the physico-chemical properties of the two materials. However, as can be seen from the total coliform, ammonium, and phosphate removal data, as well as Figure 6.11, these differences become less significant with time.

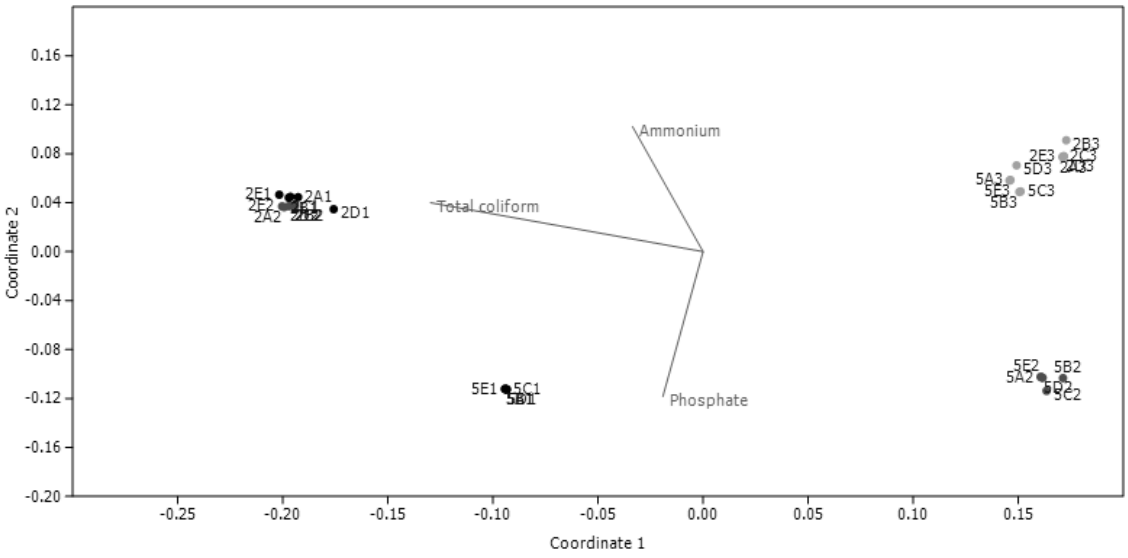

Figure 6.11 Reactor 2 (25% cocopeat) and Reactor 5 (100% cocopeat) at each depth and time point with three environmental factors: ammonium, phosphate, and total coliform. Data labeled wetland number, depth, and time point. Depths A – E were surface, 0.153 m, 0.28 m, 0.33 m, and 0.55 m, respectively. Time 1 – 3 were 49, 92, and 169 d respectively.

Impacts of Depth. At the initial sampling point (Day 46), there was a significant correlation with all three environmental factors at depth A (Figure 6.12). In general, ammonium had little effect on communities at depth A. We hypothesize this is due to the steady state ammonium removal observed starting at the first sampling day (Day 24). The ammonium and phosphate axes are inversely correlated due to redox zone community differences (aerobic versus anaerobic). In contrast to ammonium, we observed a greater effect on microbial communities by phosphate and total coliform. As time progresses, phosphate distinction is smaller at depth A, likely due to the decreasing amounts of phosphate leaching (Figure 6.5). Total coliform likely contributes to community

differences, as we hypothesize the total coliform removal happens in the upper portions of the wetland due to the effects of biofilm. Biofilms are most likely to form in the upper depths of the wetland where nutrient and carbon concentrations will be the highest, as these are nutrients supporting biofilm establishment (Chandy et al, 2001; Delille et al, 2007). Communities slowly acclimated and converged at depth A, but eventually clustering of all reactor communities was observed at Day 169.

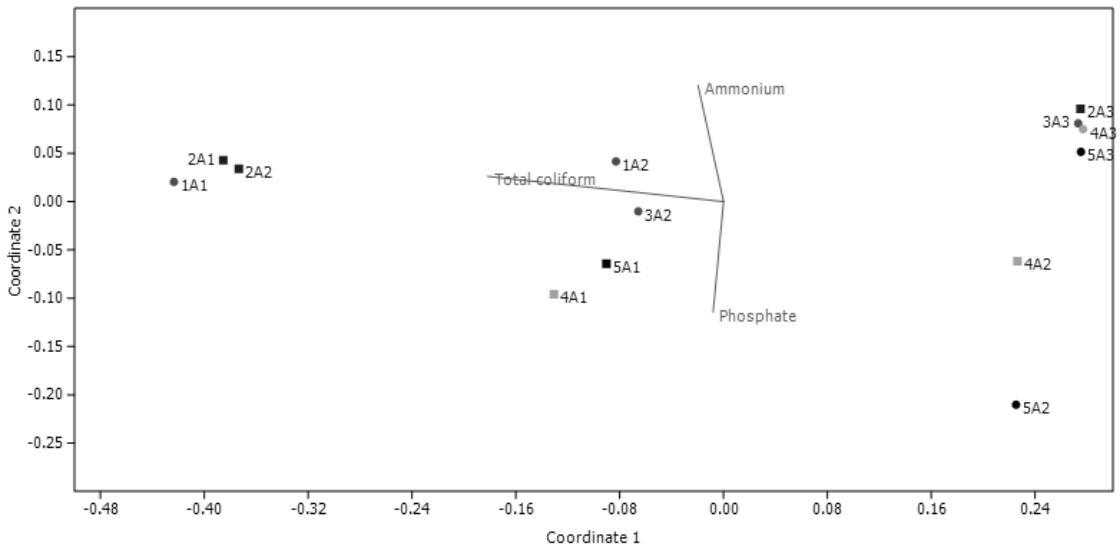

Figure 6.12 Each reactor at depth A (surface). Data labeled wetland number, depth, and time point. Wetlands 1 – 5 were 0%, 25%, 50%, 75%, and 100% cocopeat, respectively. Time 1 – 3 were 49, 92, and 169 d respectively.

Figure 6.13 shows community shifts based on depths B, C, D, and E. The ammonium and phosphate axes are switched from depth A (Figure 6.10), possibly indicating a switch from an aerobic zone to an anoxic or anaerobic zone. Total coliform

becomes less important over depth, likely because total coliform is removed by biofilm in the upper portions of the constructed wetland where nutrient and carbon concentrations are highest, as these are essential for a growing biofilm (Chandy et al, 2001; Delille et al, 2007). Additionally, coliform are known to be facultative anaerobes, implying anaerobic treatment alone will not remove them, since they can survive in anaerobic conditions (Feng et al, 2002). This supports our hypothesis that coliform removal is likely due to predation within a biofilm. The most spread is observed in time point 1 and 2. Time 3 is clustered. Coliform is independent of the matrix since it is on the horizontal axis, but community development and hence nutrient removal is correlated.

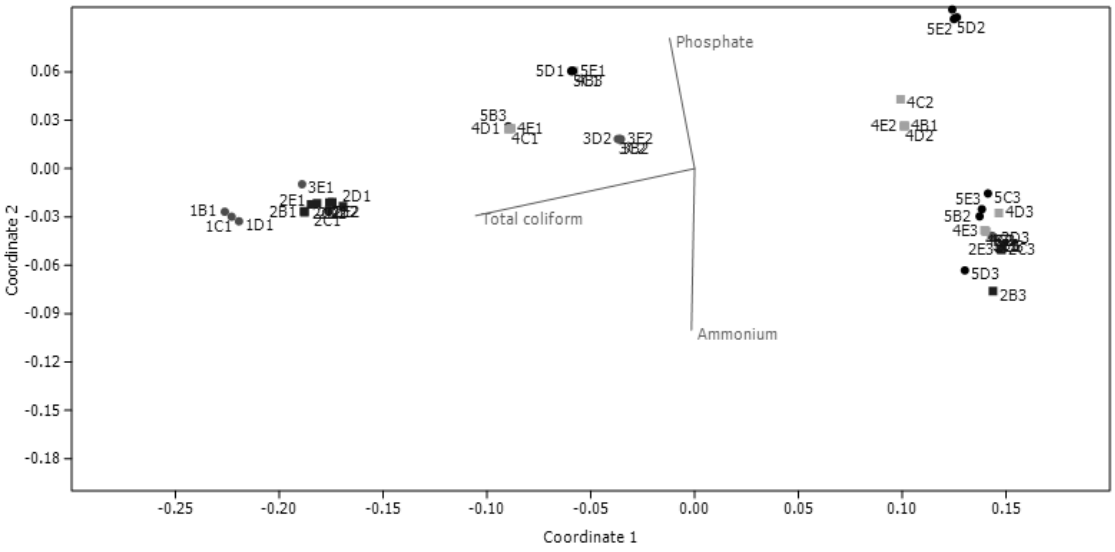

Figure 6.13 Each reactor at depth B, C, D, E (0.153 m, 0.28 m, 0.33 m, and 0.55 m, respectively). Data labeled wetland number, depth, and time point. Wetlands 1 – 5 were 0%, 25%, 50%, 75%, and 100% cocopeat, respectively. Time 1 – 3 were 49, 92, and 169 d respectively.

6.5 Conclusions

Cocopeat appears to be a suitable material for total coliform, phosphate, and ammonium removal. Based on the T-RFLP data, we observe that the microbial community stabilizes more efficiently with higher ratios of cocopeat. Reasons for this observation are previous existence of strong microbial community due to high carbon level and increased surface area as compared to gravel. As the microbial community in each wetland stabilized, we observed a decrease in total coliform concentration. Wetlands with increasing amounts of cocopeat had greater ammonium removal than wetlands with greater gravel amounts. With increased amounts of cocopeat, we observed increasing concentrations of phosphate in the effluent due to leaching, but after 100 days, leaching was no longer apparent. We did not observe complete removal of ammonium or phosphate in the constructed wetlands, which proved beneficial for the ornamental plants growing in the secondary treatment step (Figures C6 – C11). Not only did the ammonium and phosphate increase ornamental plant growth, but the ornamental plants provided additional removal of these two contaminants in most cases.

Overall, this setup is an effective technique to treat municipal wastewater in tropical, rural areas where wastewater treatment is not currently available. In lab-scale column reactors without plants, cocopeat was able to accomplish nitrification and denitrification (Chapter 5). Here, we show the importance of plants for the removal of nitrate and the promotion of an anaerobic zone and further removal of phosphorus.

Chapter 7. Conclusions and Engineering Significance

The goal of this dissertation work was to contribute to the body of knowledge on specific point-of-use water and wastewater technologies. The biosand filter was studied in both lab and field conditions and it was found that total coliform is not a reliable indicator for *Vibrio cholerae*, total coliform and *V. cholerae* experienced different removal efficiencies within the biosand filter, and that there are several factors controlling biosand filter performance, including idle time, TOC, filter time in operation, physical/chemical attachment, and schmutzdecke composition, as well as behavior factors. Cocopeat was studied for its ability to promote nitrification and denitrification in lab-scale vertical flow columns treating simulated wastewater. Cocopeat achieved similar levels of nitrification and denitrification compared to traditional packing media. Finally, cocopeat packed vertical flow constructed wetlands were operated in Vietnam for the treatment of septic tank effluent. This setup proved effective for the removal of nitrogen, phosphorus, and total coliform in the treatment of wastewater. A summary of key findings for each objective as well as the engineering significance of this dissertation research are described below.

7.1 Key findings

Conclusion 1: Total coliform are not appropriate indicator organisms to estimate concentration and removal efficiency of *V. cholerae* in biosand filters.

As shown in Chapter 3, total coliform consistently underestimated *V. cholerae* concentration in source waters and consistently underestimated *V. cholerae* removal efficiency in biosand filters. This is significant as total coliform may underestimate other pathogens of interest, including *Shigella* and *Salmonella*, and may not always underestimate removal efficiencies. Conversely, it may also overestimate them, which could also lead to uninformed decisions about the need for treatment, although this argument would need to be tested in a much more fundamental way than with simple one-organism correlations. This indicates a need for field-ready pathogen-specific tests that can analyze concentrations, especially in an outbreak situation where the concentrations of a specific pathogen are of interest. In Chapter 3, we describe the development and effectiveness of a field-ready *V. cholerae* specific test to measure concentration.

Conclusion 2: Parameters controlling biosand filter performance include: Total organic carbon (TOC) loading, schmutzdecke composition, time in operation, idle time, and physical/chemical attachment.

In Chapter 3 and 4, different parameters controlling biosand filter performance were explored. In Chapter 3, idle time, time in operation and amount of EPS were shown to be critical to biofilm microbial community shifts. Also, as time in operation increased,

removal efficiency of *V. cholerae* increased, with an apparent steady state after three months of operation. In Chapter 4, TOC loading was found to be critical to *V. cholerae* removal. The highest removal efficiencies were observed in the biosand filter receiving the lowest TOC. The schmutzdecke composition was also influenced by TOC, both in terms of microbial community structure and amount of EPS. Finally, physical/chemical attachment was also shown to be important, especially at low TOC loadings.

Conclusion 3: Physical/chemical attachment is critical during startup. Physical/chemical attachment plays a more critical role than schmutzdecke effects in filters receiving low TOC while schmutzdecke effect is more important than physical/chemical attachment in filters receiving high TOC.

In Chapter 4, the effects of schmutzdecke and physical/chemical attachment were analyzed separately and physical/chemical attachment was shown to be important during startup, as the new sand was able to achieve almost complete removal of *V. cholerae* without schmutzdecke. Furthermore, it was shown that the sand that received low TOC waters had a higher attachment coefficient than did the sand that received high TOC water, indicating that the sand receiving low TOC water facilitated more physical/chemical attachment of *V. cholerae* than did the sand receiving high TOC water. The high TOC biosand filter also had significantly more EPS than did the biofilm from the low TOC biosand filter, indicating that schmutzdecke effects were more important in the high TOC filter.

Conclusion 4: Cocopeat is comparable to traditional packing media and can successfully accomplish nitrification and denitrification in the treatment of synthetic wastewater.

For the setup outlined in Chapter 5, the most efficient nitrification and denitrification was observed in phase III – where a shallow aerobic zone and a deep anoxic zone were promoted. A deeper aerobic zone is required to further reduce ammonium. An anaerobic zone needs to be incorporated in order to promote microbial degradation of phosphorus. Overall, cocopeat accomplished similar removal of BOD as sphagnum peat, more efficient anoxic conditions based on DO, and had less acidic conditions than sphagnum peat. Cocopeat supported nitrification and denitrification of simulated wastewater, but phosphorus removal was not accomplished likely due to the short hydraulic residence time. Cocopeat performed comparably to sphagnum peat, a well-documented traditional packing medium for biofilters treating wastewater, and can achieve long-term operation.

Conclusion 5: Cocopeat can be used successfully as a packing medium in constructed wetlands treating wastewater for the removal of nitrogen, phosphorus, and total coliform.

Cocopeat and gravel packed wetlands were operated in Vietnam for the treatment of wastewater. At steady state, each wetland had significantly lower phosphate concentrations than the influent. Ammonium removal was greater than 95% in each wetland containing cocopeat throughout the course of study, but was significantly higher in the wetland without cocopeat, indicating that cocopeat was critical for nitrification. We did not observe complete removal of ammonium or phosphate in the constructed

wetlands, which proved beneficial for the ornamental plants growing in the secondary treatment step. Not only did the ammonium and phosphate increase ornamental plant growth, but the ornamental plants provided additional removal of these two contaminants.

7.2 Recommendations for biosand filter operation

The results from this dissertation suggest that total coliform concentration in water should not be used as an indication of *V. cholerae* concentration, nor should total coliform removal efficiency be used as an indication of *V. cholerae* removal efficiency in biosand filters. Tests measuring indicator organism presence/absence or concentration should not replace field-ready pathogen specific tests, when the target organism is a specific pathogen, such as in an outbreak situation. It is interesting to note that *V. cholerae* and total coliform had different removal efficiencies within the biosand filter. This may suggest that there are different removal mechanisms for different bacteria or that bacteria surface properties are critical for biofiltration removal efficiency. Additionally, time in operation was found to be critical to filter performance. In field biosand filters, there was no statistical difference in filter performance after filters had operated for one month. In lab biosand filters, steady state removal efficiencies were reached after 42 charges. Although water quality may improve after just a few charges, we also observed that water quality may worsen before the reactor reaches steady state, so it has been recommended to use chlorine after filtration before consumption. Care should be taken to never use chlorine prior to adding water to the filter as the chlorine

will negatively impact the schmutzdecke. Although, chlorine would help disinfect the water whether added before or after biofiltration, so it cannot really make water quality (the objective) worse, unless one destroys the schmutzdecke and then stops using chlorine. Otherwise, chlorine plus biosand filter use would be better than chlorine only or biosand only since there are multiple removal mechanisms in the combination (even without the schmutzdecke). In addition, source water characteristics may have a large impact on biosand filter performance. In particular, TOC was found to have a direct impact on biosand filter operation. The higher the TOC concentration, the lower the observed removal efficiency. This suggests that users should always use the cleanest water available. This finding also draws attention to one of the many factors that could be complimenting the results observed in the Haiti study. There are many behavioral factors that can affect biosand filter performance and confound treatment efficacy data, including amount of water passed through the filter, idle time, how often the filter is cleaned (schmutzdecke disturbance), and the use of multiple water sources. Because of these factors, and others, and the length of time required to reach steady state, biosand filters may not be appropriate in an emergency situation. Finally, biofilm shearing can occur in biosand filters. Care should be taken when pouring water into the filter. Diffuser plates should always be employed, and the diffuser plates should have a tight fit, thoroughly covering the biofilm, as the crashing water could cause biofilm to shear and reduce EPS, thereby reducing the effectiveness of treatment.

7.3 Recommendations for cocopeat-packed biofilters treating wastewater

Cocopeat is a suitable packing medium and may be desirable over traditional packing medium such as sphagnum peat, because it does not need to be mined. In locations such as Southeast Asia, cocopeat is readily available and relatively inexpensive, potentially making cocopeat a desirable medium for point-of-use wastewater treatment. In this study, the wetland packed with cocopeat only showed the highest ammonium removal efficiency, but also had the highest amounts of phosphate in the effluent throughout the study. Cocopeat has been shown to leach phosphate into waters, so if the peat can be thoroughly washed in water prior to usage, this should improve treatment efficiency. Alternatively, a mix of cocopeat and gravel could be used. Here, we showed that a mix of 75% cocopeat and 25% gravel or a mix of 50% cocopeat and 50% gravel was effective at both ammonium and phosphate reduction. Additional, improvements to the cocopeat biofiltration process may be made with respect to surface area. If the peat can be processed further to make it more fine, this would provide more surface area, potentially increasing biological activity and treatment efficiency. We recommend using planted constructed wetlands as nitrate is the nitrogen species most available to plants. In our system, nitrate concentrations were very low, which may have allowed the promotion of an anaerobic zone, which allows for microbial degradation of phosphate. Since this system may not accomplish full removal of nitrogen and phosphate, we recommend using the effluent from the constructed wetland for agricultural or aquacultural purposes, such as growing ornamental plants or nourishing fish farms. Lastly, in this setup, there was a

space requirement to allow room for the peat-packed constructed wetland, as well as the ornamental planters for secondary treatment. Further research is needed to determine if this system could be adapted for dense, urban areas.

7.4 Engineering significance and future work

Overall, this dissertation work shows that biosand filters may be an efficient point-of-use drinking water treatment, if designed and implemented correctly. The potentially important parameters for treatment success are time in operation and concentration of TOC. As point-of-use water treatment continues to be encouraged in areas where municipal water treatment is not available, it is important to critically evaluate the available technologies before they are promoted to users. Here, we have further characterized the biosand filter and can confirm that they can effectively remove *V. cholerae* from water, given enough time in operation and proper operation. Future research is needed to further investigate the optimal idle time and how this will vary with influent water characteristics.

Finally, cocopeat achieves comparable nitrification and denitrification levels as traditional packing media and can be used as an appropriate biofiltration packing medium when operated in vertical flow constructed wetlands. As point-of-use wastewater treatment grows in popularity for people without access to municipal wastewater treatment, it is important to provide incentive, as wastewater technologies can be expensive and paybacks minimal. This system provides users with a constructed wetland that could embellish a yard or rooftop garden, and provides semi-treated water for use in

ornamental planters or aquaculture ponds, where the remaining nutrients can provide benefits. Future research includes evaluating performance and user acceptance of cocopeat-packed constructed wetlands in the Mekong Delta, as well as the evaluation of other biofiltration setups using cocopeat as a packing medium.

Appendix A - Chapter 3 Supplementary Information

EPS standard curves.

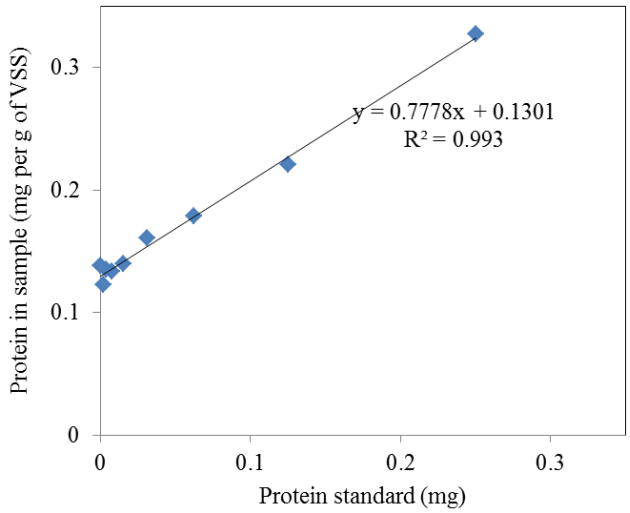

Figure A1. EPS protein standard curve

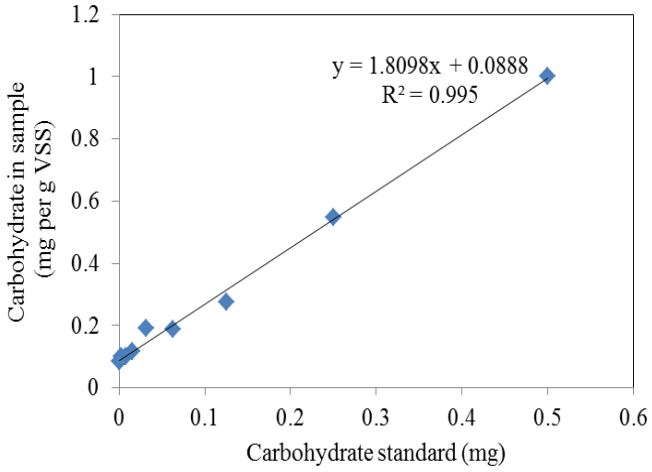

Figure A2. EPS carbohydrate standard curve

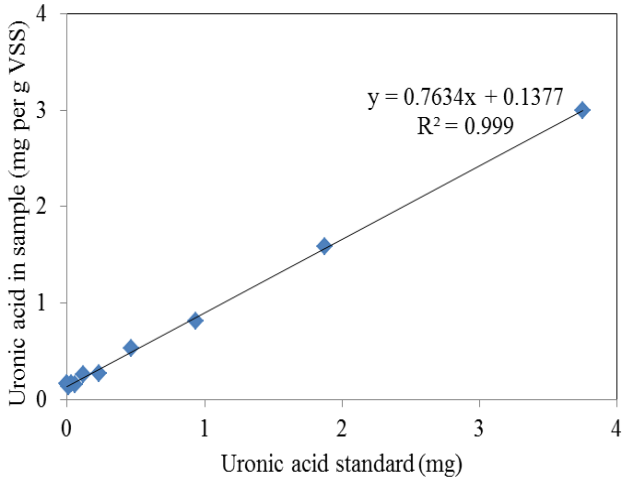

Figure A3. EPS uronic acid standard curve

Appendix B - Chapter 5 Supplementary Information

Nutrient standard curves.

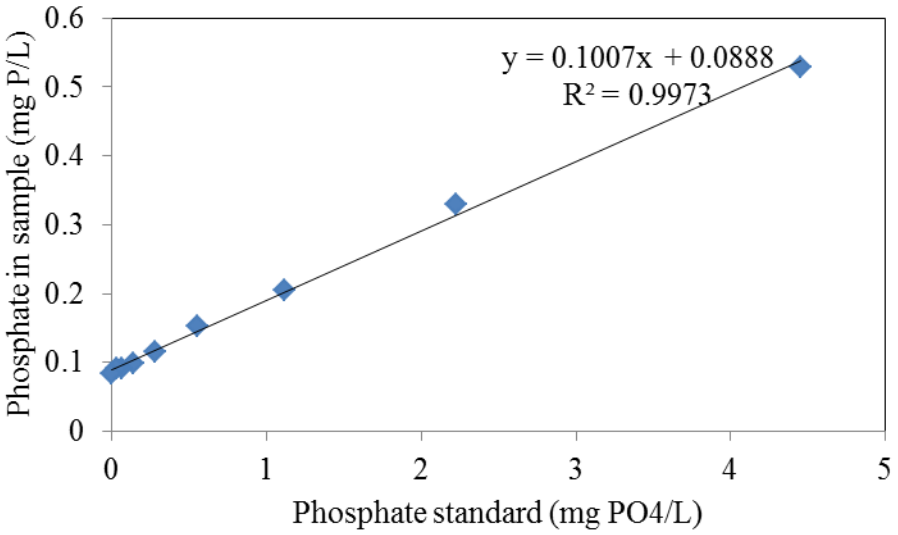

Figure B1. Phosphate standard curve

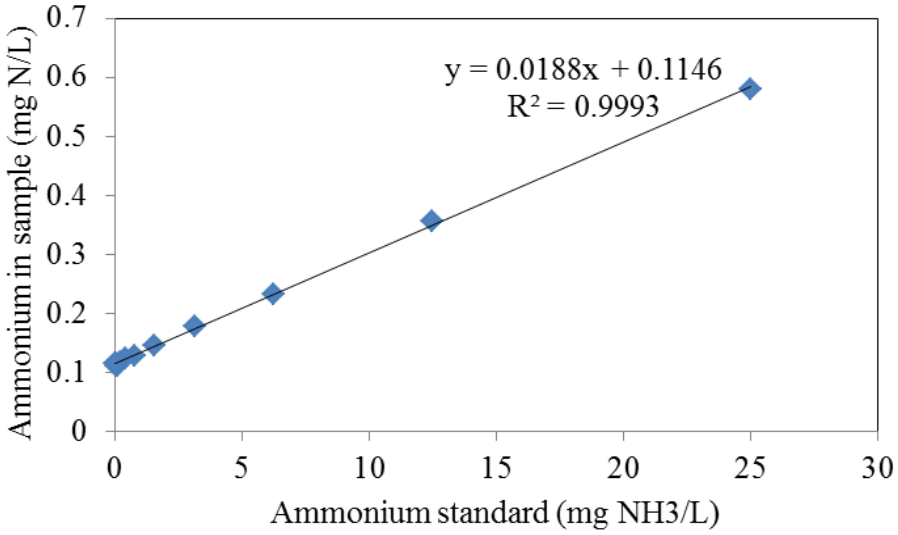

Figure B2. Ammonium standard curve

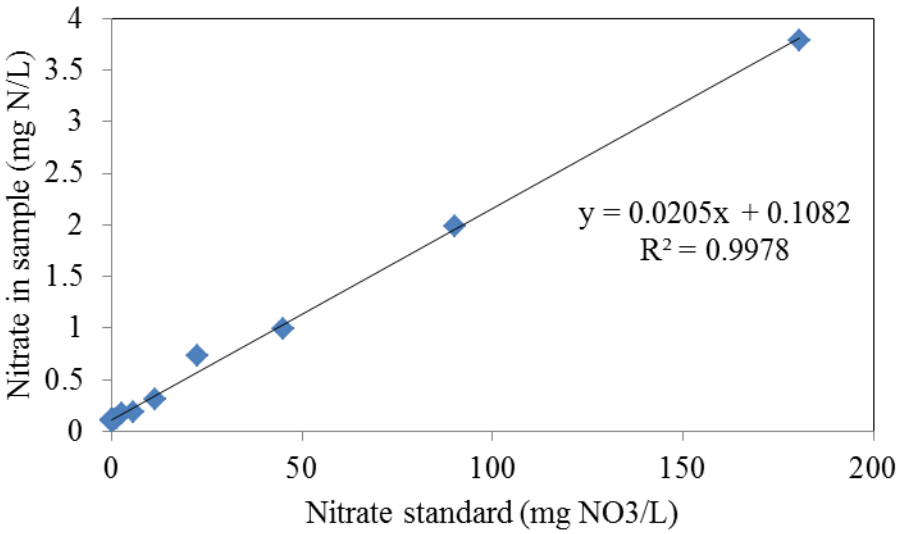

Figure B3. Nitrate standard curve

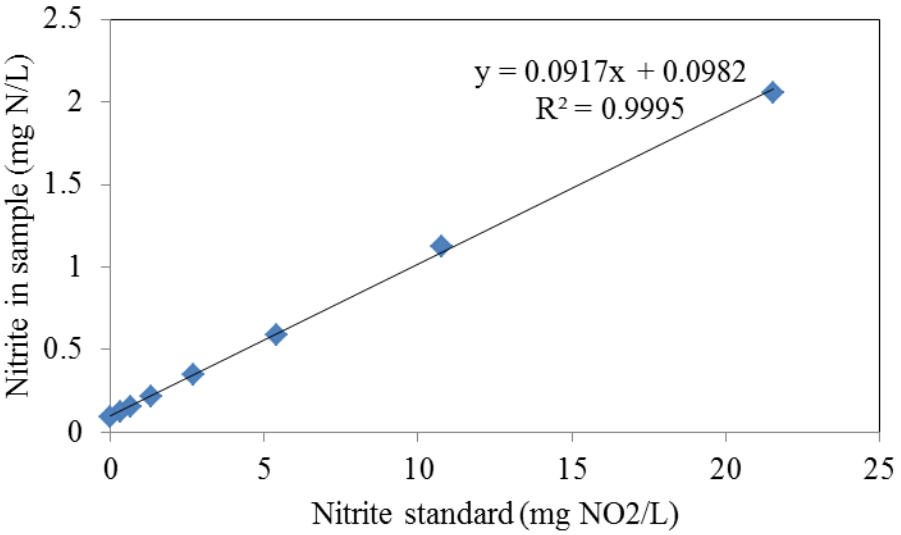

Figure B4. Nitrite standard curve

Nm-MDS linear regression – Phase I.

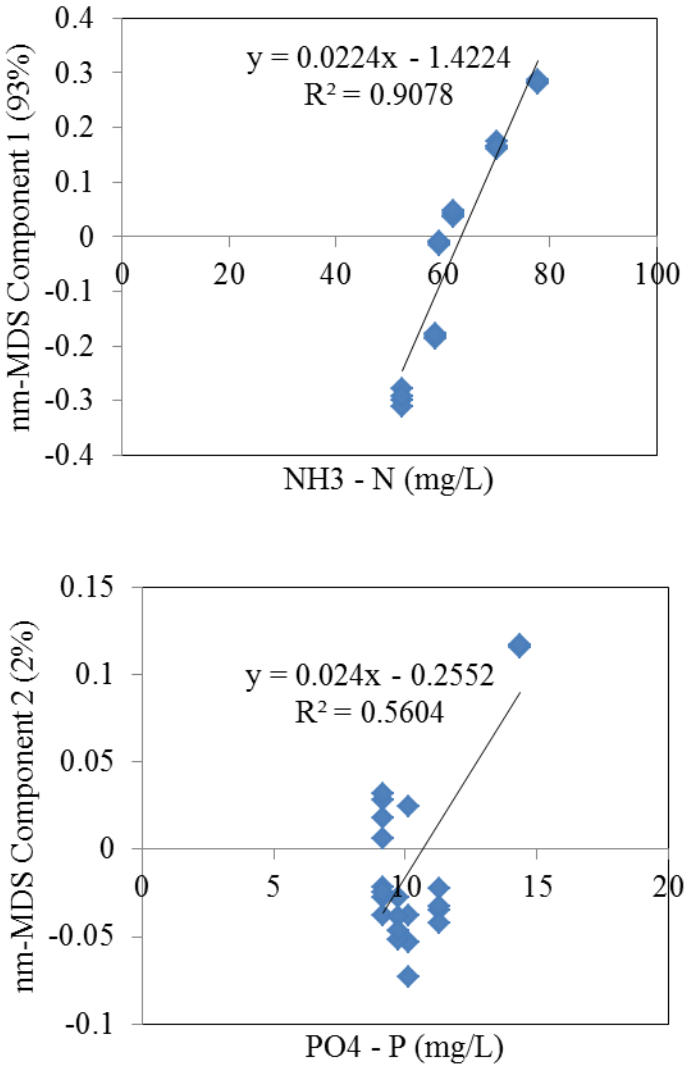

Figure B5. Phase I nm-MDS linear regressions fitting environmental factors with nm-MDS components

Nm-MDS linear regression – Phase II.

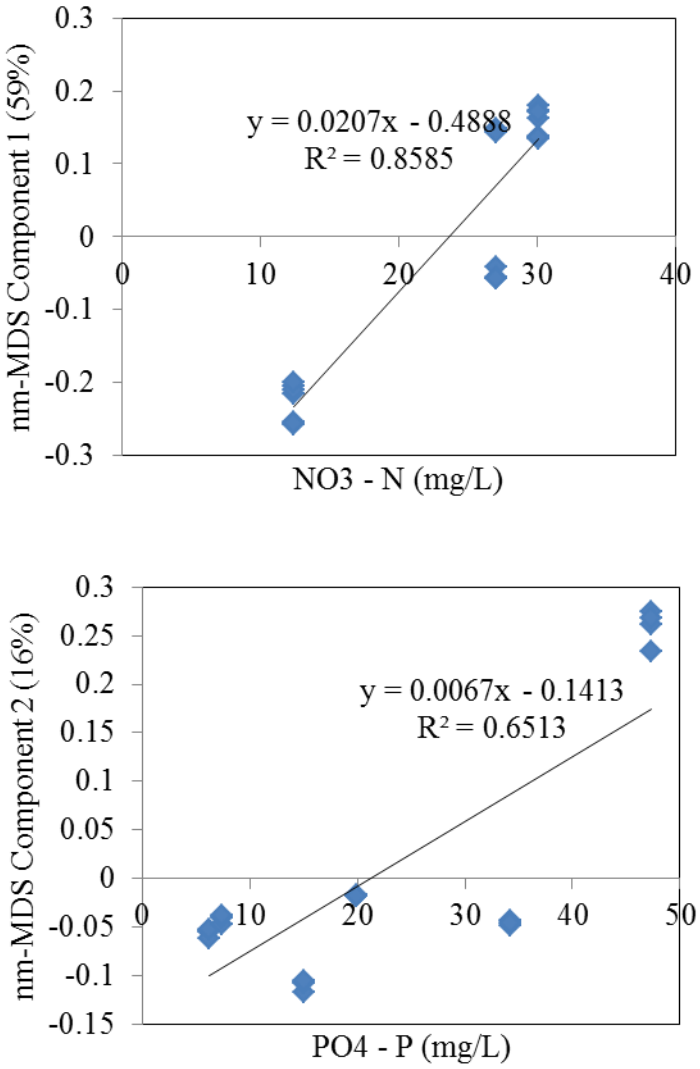

Figure B6. Phase II nm-MDS linear regressions fitting environmental factors with nm-MDS components

Nm-MDS linear regression – Phase III.

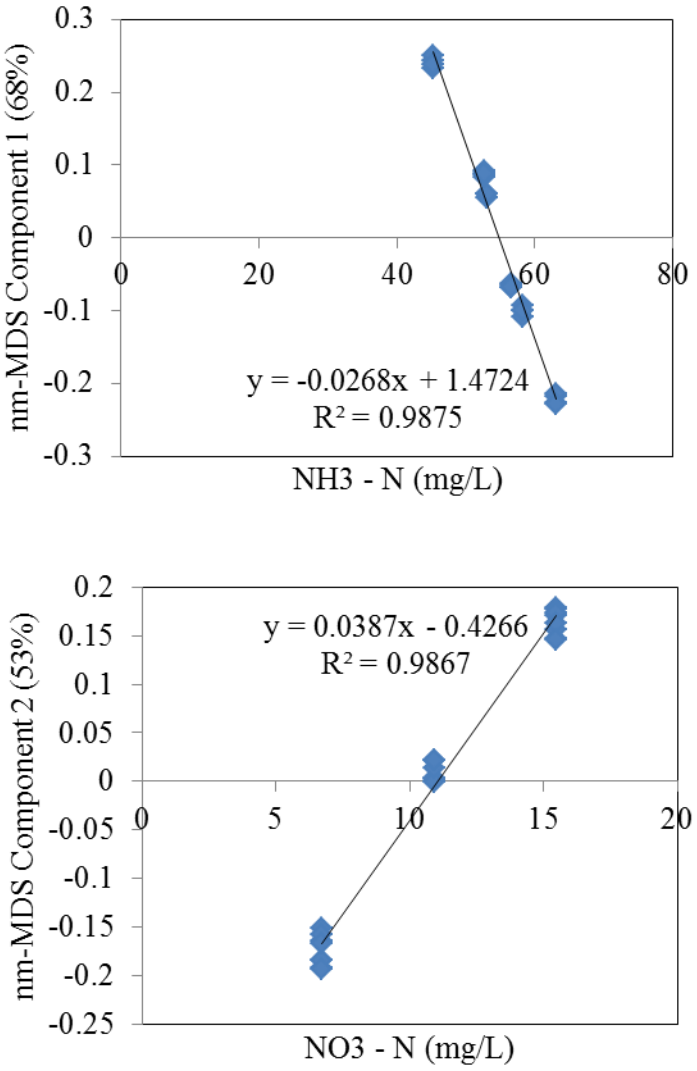

Figure B7. Phase III nm-MDS linear regressions fitting environmental factors with nm-MDS components

Nm-MDS linear regression – Phase IV.

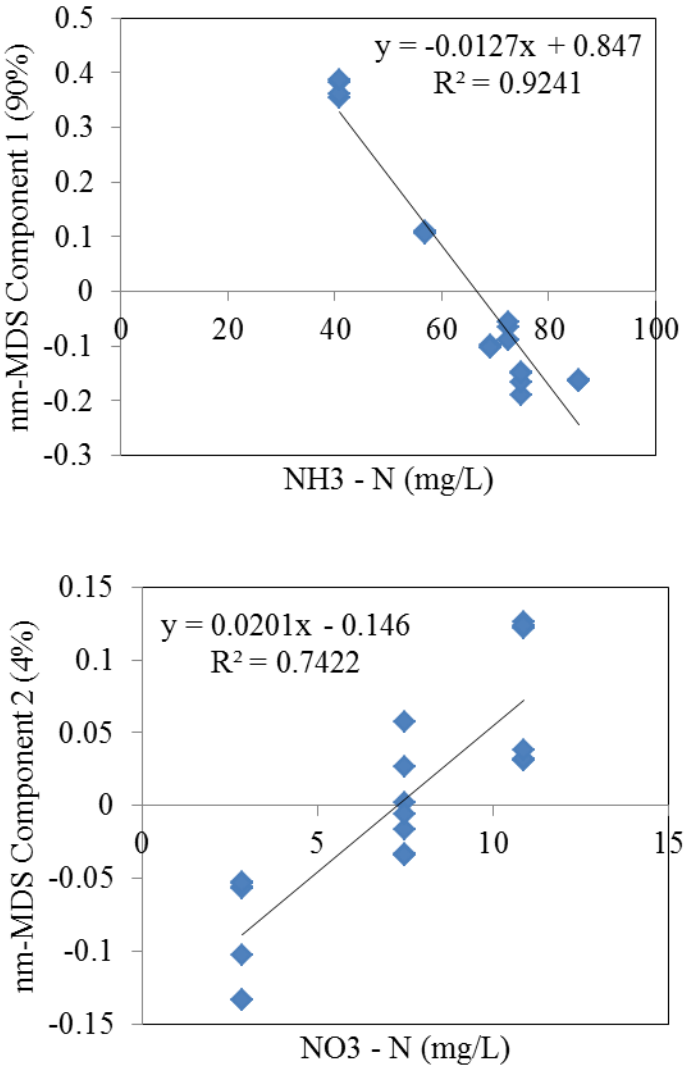

Figure B8. Phase IV nm-MDS linear regressions fitting environmental factors with nm-MDS components

Appendix C - Chapter 6 Supplementary Information

Canna indica(peat and gravel constructed wetlands) plant information.

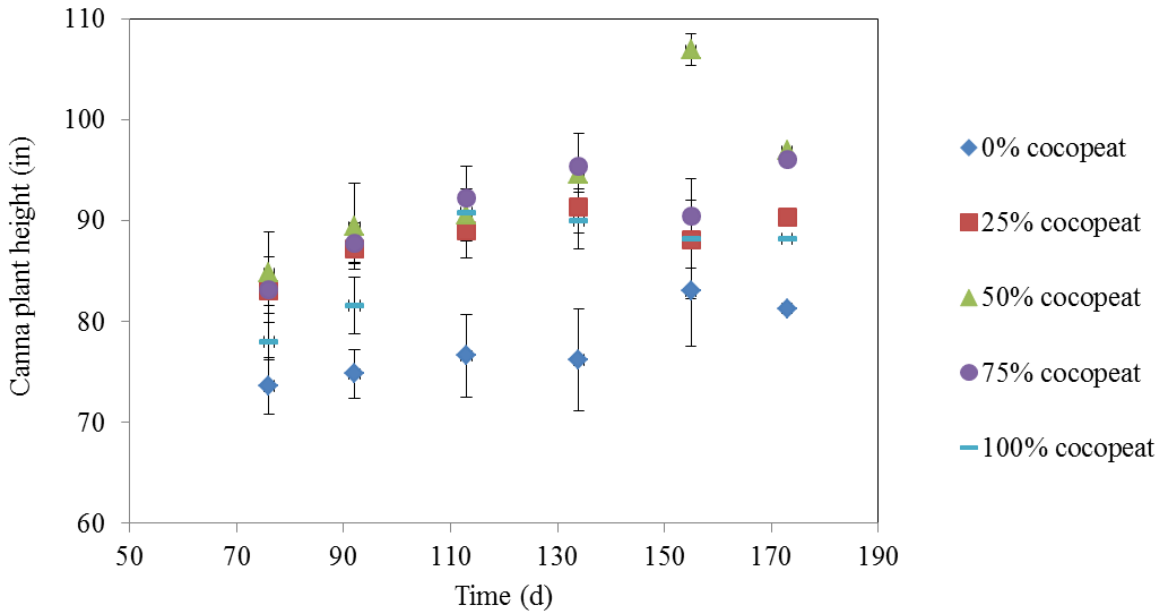

Figure C1. *Canna* plant height (in) vs time (d). Error bars represent standard deviation between the five tallest *Canna* plants.

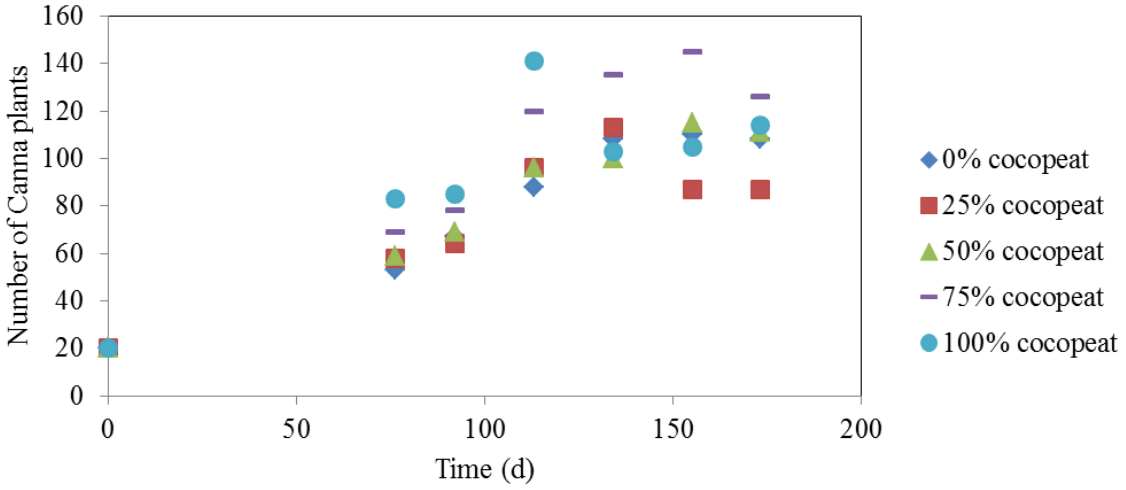

Figure C2. Number of *Canna* plants in each wetland vs time (d).

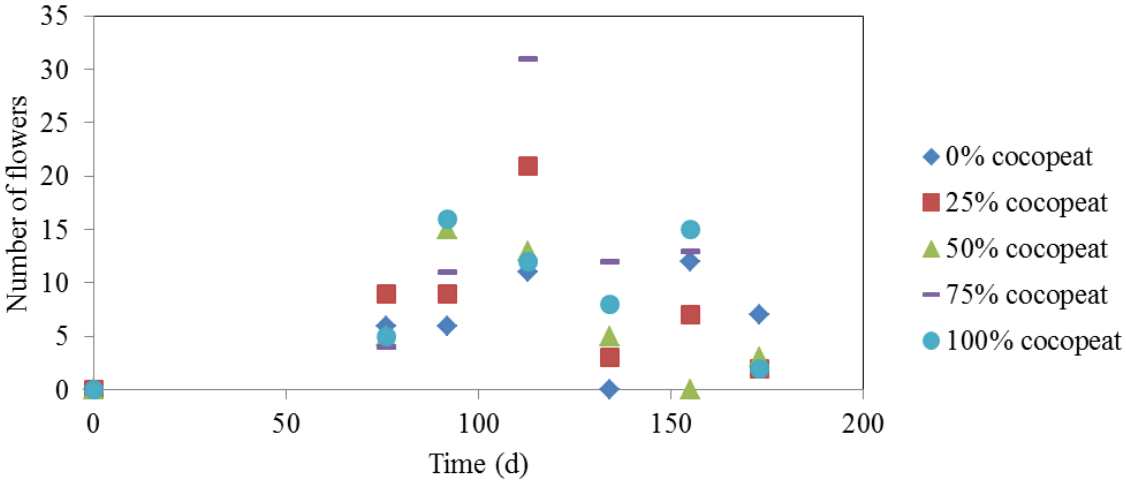

Figure C3. Number of *Canna* flowers in each wetland vs time (d).

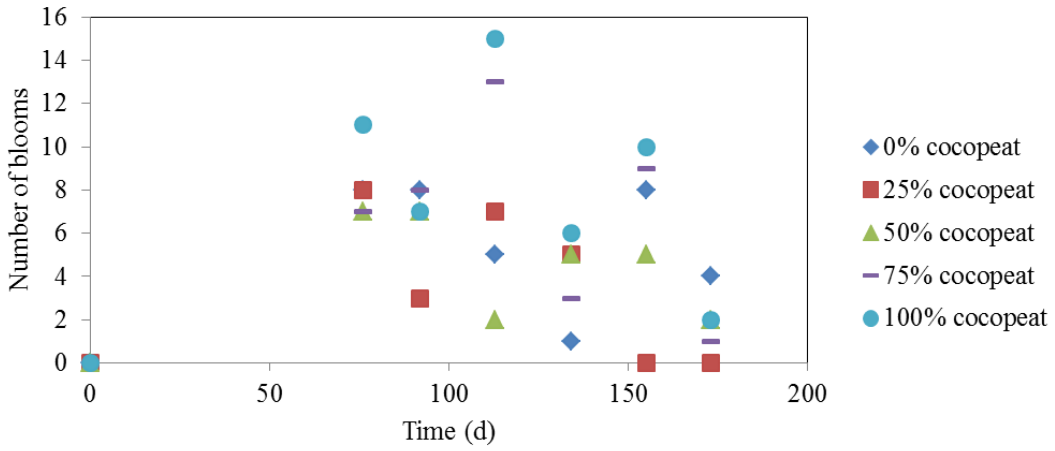

Figure C4. Number of *Canna* blooms in each wetland vs time (d).

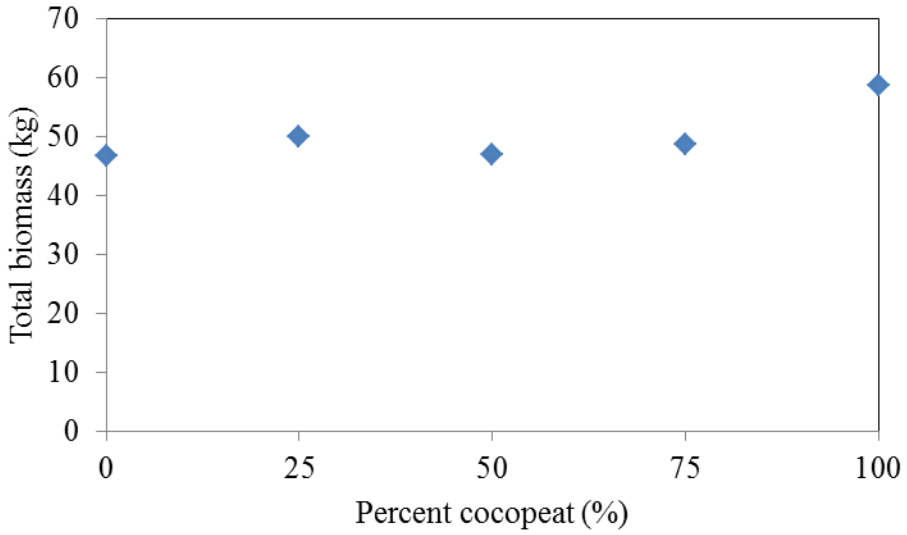

Figure C5. *Canna* biomass in each wetland at the end of the study (d = 169).

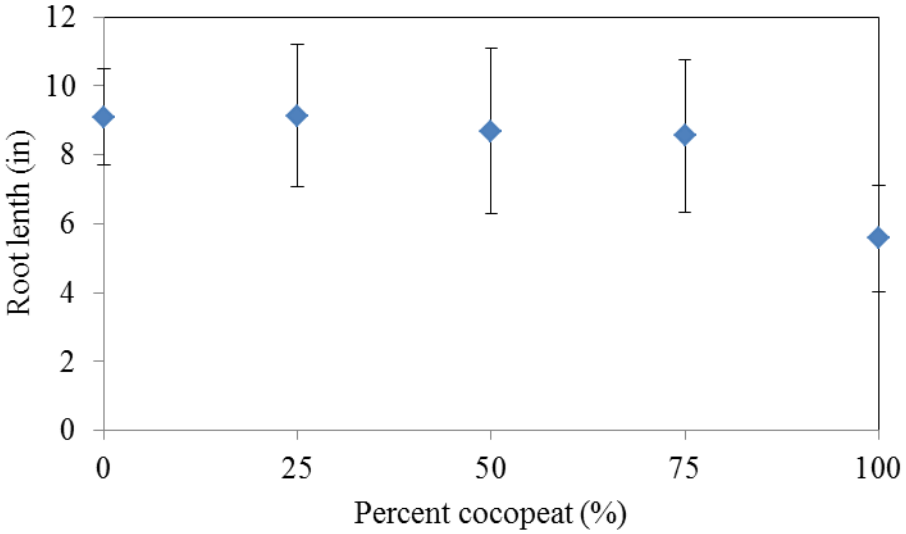

Figure C6. *Canna* root length in each wetland at the end of the study (d = 169). Error bars represent standard deviation between duplicate planters.

Caladium bicolor (ornamental planters) plant information.

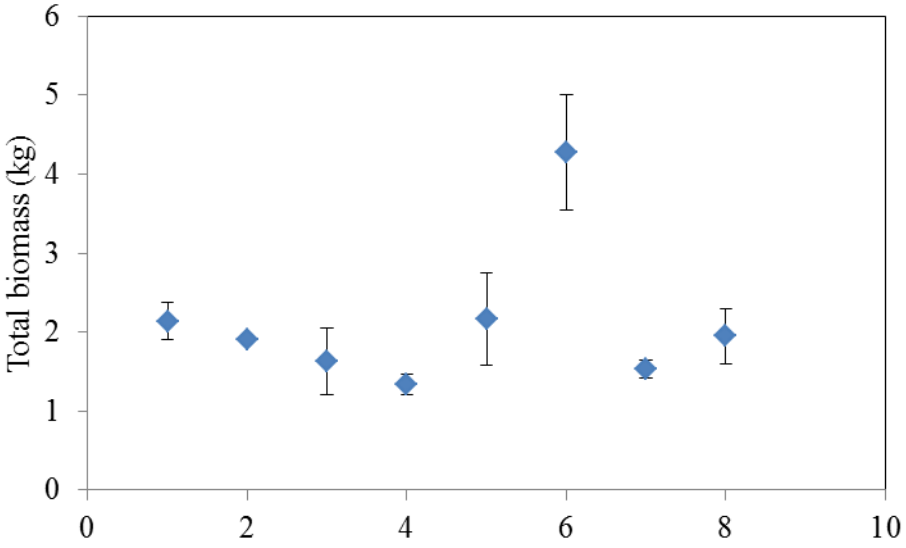

Figure C7. *Caladium* biomass in each planter at the end of the study (d = 169); planter receiving water from the 0% (1), 25% (2), 50% (3), 75% (4), and 100% (5) cocopeat constructed wetlands. Also, control planters receiving wastewater (6), tap water (7), and no water except from rain events (8). Error bars represent standard deviation between duplicate planters.

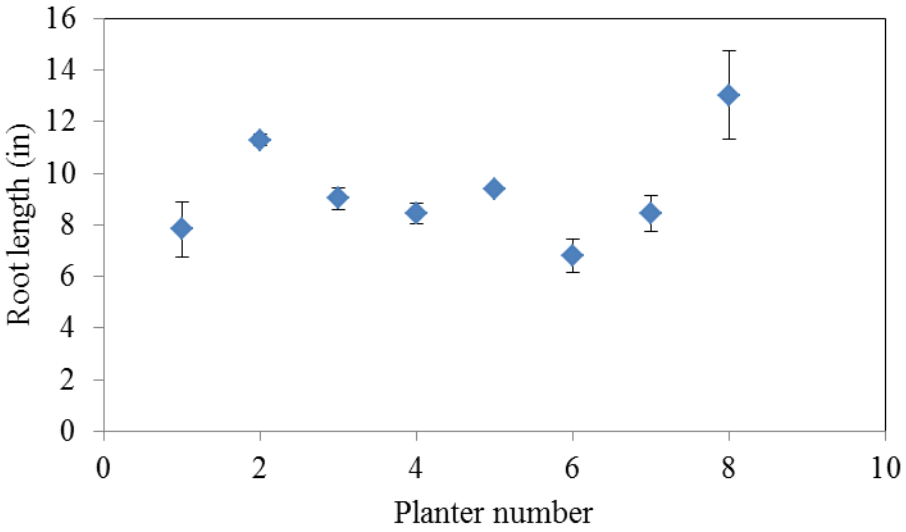

Figure C8. *Caladium* root length in each planter at the end of the study (d = 169); planter receiving water from the 0% (1), 25% (2), 50% (3), 75% (4), and 100% (5) cocopeat constructed wetlands. Also, control planters receiving wastewater (6), tap water (7), and no water except from rain events (8). Error bars represent standard deviation between duplicate planters.

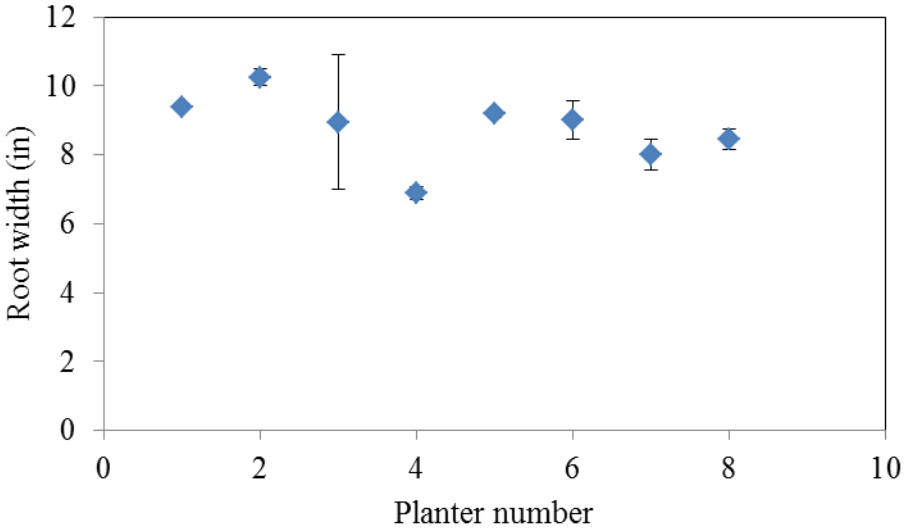

Figure C9. *Caladium* root width in each planter at the end of the study (d = 169); planter receiving water from the 0% (1), 25% (2), 50% (3), 75% (4), and 100% (5) cocopeat constructed wetlands. Also, control planters receiving wastewater (6), tap water (7), and no water except from rain events (8). Error bars represent standard deviation between duplicate planters.

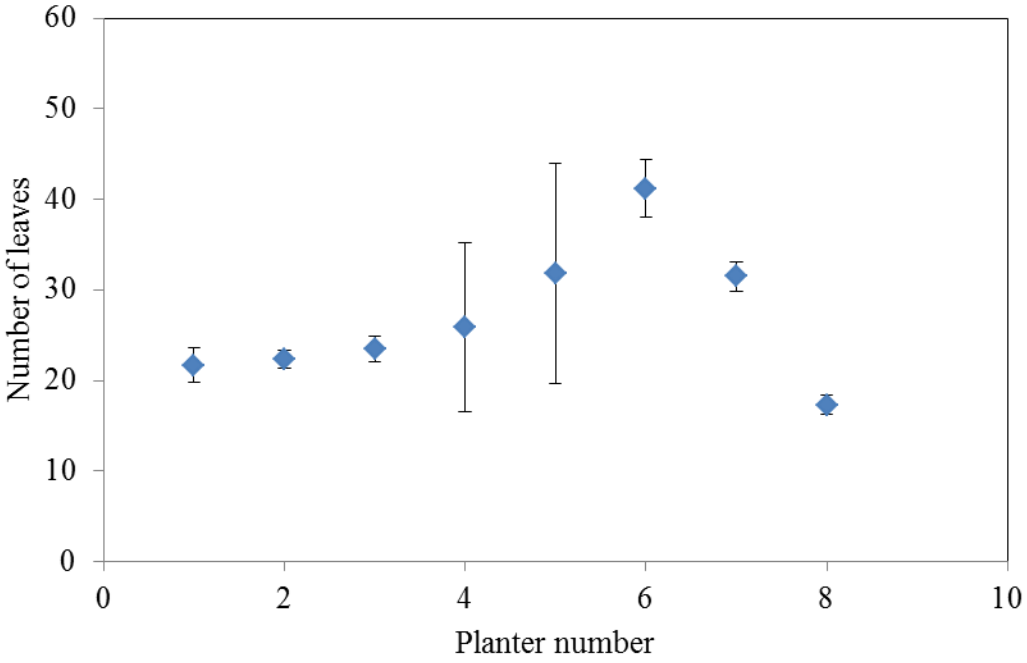

Figure C10. *Caladium* leaf number in each planter at the end of the study (d = 169); planter receiving water from the 0% (1), 25% (2), 50% (3), 75% (4), and 100% (5) cocopeat constructed wetlands. Also, control planters receiving wastewater (6), tap water (7), and no water except from rain events (8). Error bars represent standard deviation between duplicate planters.

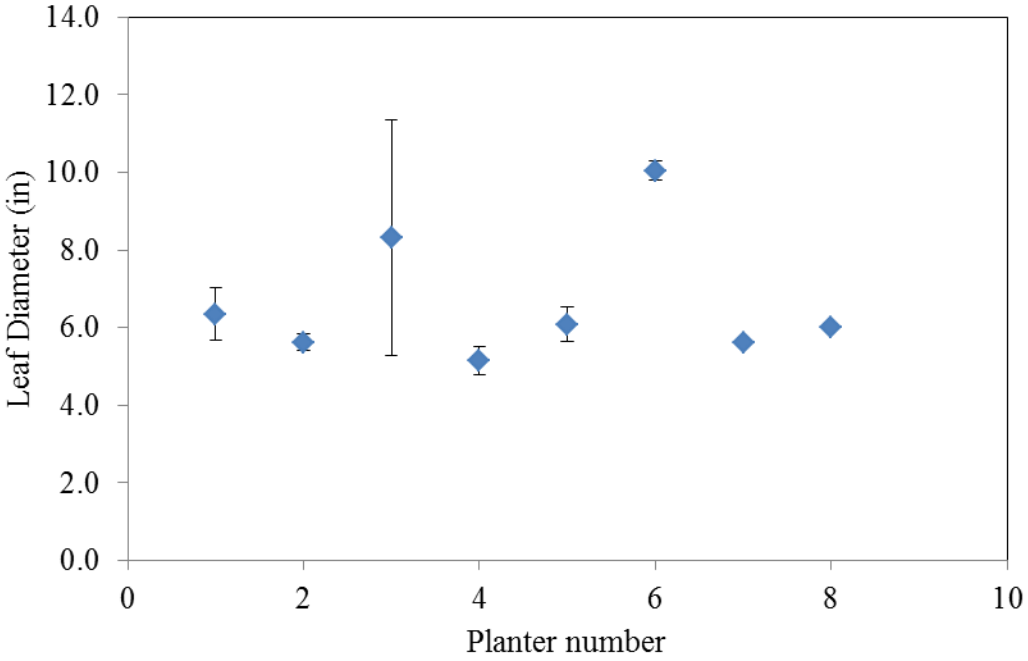

Figure C11. *Caladium* leaf diameter in each planter at the end of the study (d = 169); planter receiving water from the 0% (1), 25% (2), 50% (3), 75% (4), and 100% (5) cocopeat constructed wetlands. Also, control planters receiving wastewater (6), tap water (7), and no water except from rain events (8). Error bars represent standard deviation between duplicate planters.

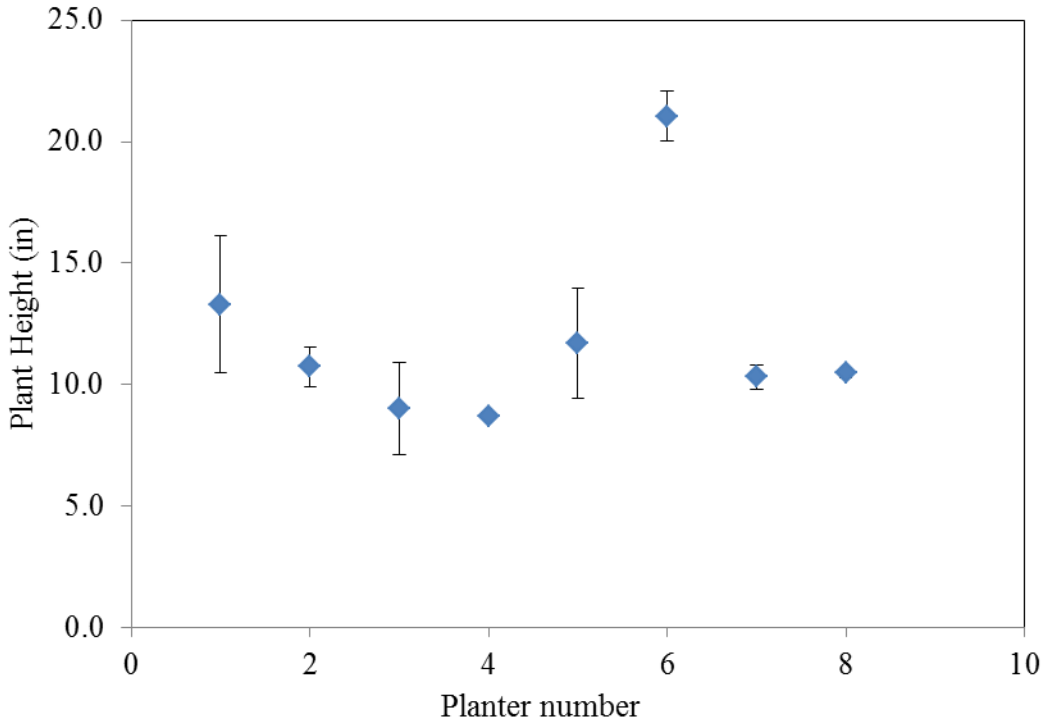

Figure C12. *Caladium* plant height in each planter at the end of the study (d = 169); planter receiving water from the 0% (1), 25% (2), 50% (3), 75% (4), and 100% (5) cocopeat constructed wetlands. Also, control planters receiving wastewater (6), tap water (7), and no water except from rain events (8). Error bars represent standard deviation between duplicate planters.

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Biography

Ashley Anne Thomson was born in Fort Myers, Florida on October 20, 1987. The universities attended and degrees obtained as well as her publications and academic honors are listed below.

EDUCATION

Duke University, Durham, NC — June 2010 – May 2014
Ph.D. Candidate in Civil and Environmental Engineering
Dissertation Project: Development of Water and Wastewater Biofiltration Technologies for the Developing World using Locally Available Packing Media: Case Studies in Vietnam and Haiti

Duke University, Durham, NC — May 2013
Certificate in International Development Policy

Florida State University, Tallahassee, FL — April 2010
Bachelor of Science in Civil Engineering, *Summa Cum Laude*
Double Major in Civil and Environmental Engineering

PUBLICATIONS

A.A. Thomson, D. Robbins, and C.H. Nguyen. Codigestion for Methane Capture and Use: Optimization for Backyard and Small Commercial Farmers in the Lower Mekong Basin. *Natural Resources and Environment towards Sustainable Development*: The First Conference on Science and Technology at the Ho Chi Minh City University of Natural Resources and Environment, Ho Chi Minh City, Vietnam (December 2012).

AWARDS AND NOTEABLE RECOGNITIONS

NSF Graduate Research Opportunities Worldwide	January 2014 – April 2014
Fulbright Scholarship in Vietnam	August 2012 – June 2013
National Science Foundation Graduate Fellow	June 2010 – May 2014
Pratt School of Engineering Fellowship Recipient	August 2010 – May 2012